

# **DEVELOPMENT OF A POXVIRUS VACCINE: THE MYXOMA VIRUS/EUROPEAN RABBIT MODEL**

**A thesis submitted for the degree of Doctor of  
Philosophy**



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## DECLARATION

The research presented in this thesis is my own original work unless otherwise stated. This work was carried out in the School of Biochemistry and Molecular Biology, Faculty of Science, at The Australian National University. The material presented in this thesis has not been submitted for any other degree.

Statistical advice was obtained from Dr Jeff Wood, Statistical Consulting Unit, The Australian National University. The embedding, sectioning and staining of fixed tissues was performed by Anne Prins at the JCSMR Histology Service.



Mathew Michael Adams

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## ABSTRACT

Myxoma virus, a poxvirus of the genus *Leporipoxvirus*, is the causative agent of the disease myxomatosis, which is highly lethal in European rabbits. Current vaccines to protect domestic rabbits against myxomatosis are not permitted in Australia due to fears the vaccine virus could be transmitted to wild rabbit populations.

This thesis has used the myxoma virus/rabbit model to determine the efficacy of novel vaccination strategies against a naturally transmitted, lethal poxvirus disease in the species at risk. Candidate vaccines were evaluated for their acceptability in domestic rabbits, their efficacy against lethal myxoma virus challenge and their non-transmissibility between rabbits.

In the first part of this thesis, the protective efficacy of DNA vaccines expressing the myxoma virus antigens M055R, M073R, M115L and M121R, and co-expressing rabbit interleukin-2 or interleukin-4 was examined. None of the vaccine constructs were able to protect rabbits from lethal myxoma virus challenge.

Live, attenuated myxoma virus vaccines were constructed by the targeted deletion of one, two or three viral immunomodulatory genes from the genome of the attenuated myxoma virus strain Uriarra. Vaccination with TKO, a virus with deletions in the genes coding for myxoma growth factor (M010L), the anti-apoptotic factor M011L and the interferon- $\gamma$ -binding protein M-T7 conferred complete protection from myxoma virus challenge, and caused only a small lesion limited to the inoculation site in mature domestic rabbits. This virus was selected for further evaluation.

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The pathogenesis of TKO was investigated to determine the degree of tissue damage that occurred in domestic rabbits during vaccination and the potential transmissibility of the vaccine virus. TKO caused only transient pathology at the inoculation site and normal skin pathology was restored by 20 dpi following an extensive infiltration of mononuclear cells that coincided with the rapid control and clearance of the virus from the skin. TKO reached transmissible titres ( $>10^7$  pfu/g) in the skin of some domestic and wild rabbits. Also, 8 and 12 week old rabbits vaccinated with TKO developed small secondary lesions following inoculation. Therefore, TKO was not considered acceptable for use as a myxomatosis vaccine in Australia.

In the last section of the thesis, a replication-deficient myxoma virus vaccine was constructed by the deletion of the host range protein M063R (Uriarra  $\Delta$ M063R*gptgus*). Vaccination with Uriarra  $\Delta$ M063R*gptgus* resulted in a small lesion at the inoculation site only, and conferred complete protection from lethal myxoma virus challenge, although some rabbits still developed mild to moderate symptoms of myxomatosis upon challenge. A boost with the same virus conferred protection from disease comparable to replication-competent vaccines.

Uriarra  $\Delta$ M063R*gptgus* was found to have the best combination of acceptability, non-transmissibility and efficacy of the tested myxomatosis vaccines, and is a candidate for further development for use in protecting Australian domestic rabbits from myxomatosis. These results also show that homologous replication-deficient poxvirus vaccines offer a safe and effective alternative vaccination method to protect animals and humans from poxvirus diseases.

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## LIST OF ABBREVIATIONS

<sup>3</sup> H	tritium
aa	amino acids
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AGO	anogenital oedema
Ap	apoptotic body
AZ	apical light zone
BGH-pA	bovine growth hormone polyadenylation signal
bp	base pairs
BSA	bovine serum albumin
BV	blood vessel
BZ	basal light zone
C	capsule
°C	Centigrade
CEV	cell-associated enveloped virion
CD	cluster of differentiation
CDC	Centres for Disease Control
CMV-IE	cytomegalovirus intermediate/early promoter
conA	concanavalin A
CPE	cytopathic effect
CRPV	cottontail rabbit papillomavirus
CS	clinical score
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CTL	cytotoxic T lymphocyte
Cx	cortex
D	dermis
DC	dendritic cell
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribose nucleic acid
dpb	days post boosting
dpc	days post challenge

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dpi	days post infection/inoculation
dsRAD	dsRNA-specific adenosine deaminase
dsRNA	double stranded RNA
DTT	dithiothreitol
DZ	dark zone
E	epidermis
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EEV	extracellular enveloped virion
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF2 $\alpha$	eukaryotic translation initiation factor 2 $\alpha$
ELISA	enzyme-linked immunosorbent assay
EV	ectromelia virus
F	fibroblast (skin histology) primary lymphoid follicle (lymph node histology)
Fd	Faradays
FITC	fluorescein isothiocyanate
<i>g</i>	gravities
GC	germinal centre
GM-CSF	granulocyte-macrophage colony stimulating factor
<i>gpt</i>	guanine phosphorybosyl transferase
<i>gptgus</i>	guanine phosphorybosyl transferase/ $\beta$ -glucuronidase fusion gene
<i>gusA</i>	$\beta$ -glucuronidase
h	hours
HA	haemagglutinin
HF	hair follicle
His	histidine
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
hpi	hours post infection
HRP	horseradish peroxidase
IEV	intracellular enveloped virion

# **Chapter 1**

## **Introduction**

## CHAPTER 1 – INTRODUCTION

Poxviruses are the cause of many economically important diseases of domestic animals, as well as one of the most damaging human diseases in history, smallpox. Although smallpox has been eradicated, its potential use as a bioweapon has renewed interest in the development of safer and more effective smallpox and poxvirus vaccines. Currently, all commercially available poxvirus vaccines are based on live, attenuated strains of virus. These are plagued with problems including a high incidence of side effects such as vaccine-induced disease, transmissibility and poor efficacy.

This project has utilised the infection of European rabbits (*Oryctolagus cuniculus*) with myxoma virus as a model to investigate the safety and efficacy of several poxvirus vaccination strategies. This model has many advantages over other poxvirus disease models as it employs a laboratory animal infected with a virulent, highly lethal and naturally transmitted poxvirus. The vaccination strategies investigated include DNA vaccines expressing single and multiple antigens, live attenuated poxvirus vaccines constructed by the deletion of key viral immunomodulatory genes, and replication-defective vaccines. As well as exploring the broader concept of poxvirus vaccination, this project has developed candidate myxomatosis vaccines to protect Australian domestic rabbits from myxomatosis.

### 1.1 THE *POXVIRIDAE*

The *Poxviridae* are a large family of highly complex, double-stranded DNA viruses which replicate entirely in the cytoplasm of insect (*Entomopoxvirinae*) or vertebrate (*Chordopoxvirinae*) cells and include a number of important human and animal pathogens

(Moss, 2002; Table 1.1). The most notable poxvirus is variola virus, causative agent of smallpox. This virus has now been eradicated from the human population after a concerted vaccination campaign (Fenner, 1977; Fenner *et al.*, 1988). However, recent fears of its re-emergence as a bioweapon (Tegnell *et al.*, 2002; Whitby *et al.*, 2002) and the emergence of the related Orthopoxvirus monkeypox virus in American rodent populations (CDC, 2003) has brought to the fore the study of poxvirus biology and the safety and efficacy of the current suite of poxvirus vaccines (CDC, 2001; Engler *et al.*, 2002; Fenner, 1989; Roos and Eckerman, 2002; Smith and McFadden, 2002).

### **1.1.1 Poxvirus structure**

Poxviruses are the largest and most complex of the animal viruses, and exist in several infective forms. Poxvirus particles are large (350 x 270 nm for vaccinia virus; Moss, 2002), brick-shaped particles, with a nucleocapsid core surrounded by one or two membranes. The closed, linear, double stranded genome (between 144 to 288 kb amongst different poxvirus species; Gubser *et al.*, 2004) consists of a conserved central region and identical inverted terminal repeats (ITRs) that contain genes with host range and immunomodulatory functions (Figure 1.1A; Moss, 2002). The genome is contained within the nucleocapsid together with viral proteins required for early transcription of viral genes, including RNA polymerase, transcription factors, capping enzyme and poly(A) polymerase (Figure 1.1B). This viral core is wrapped in a lipid-protein membrane to form the intracellular mature virion (IMV) particle, the most abundant form of poxvirus particle. Recent reports suggest that the IMV particle may possess two or more membranes (Griffiths *et al.*, 2001; Risco *et al.*, 2002). Extracellular enveloped virions (EEV; Figure 1.1C) have an extra, Golgi-derived, membrane and are released from an infected cell into the surrounding media, or can remain attached to the cell as cell-associated enveloped

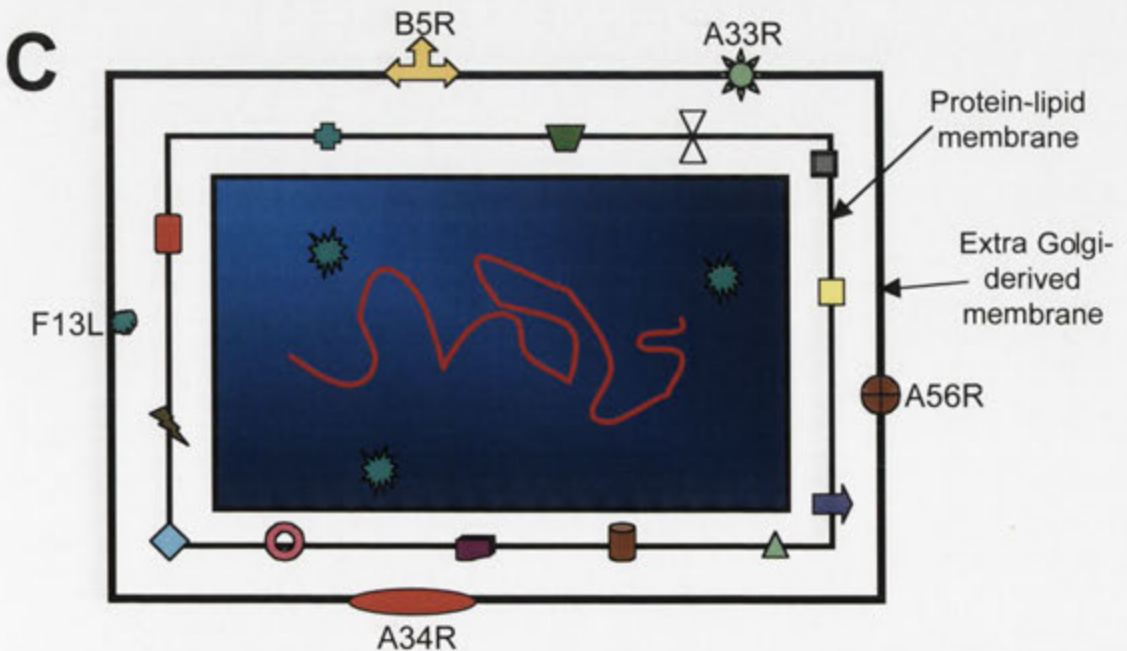
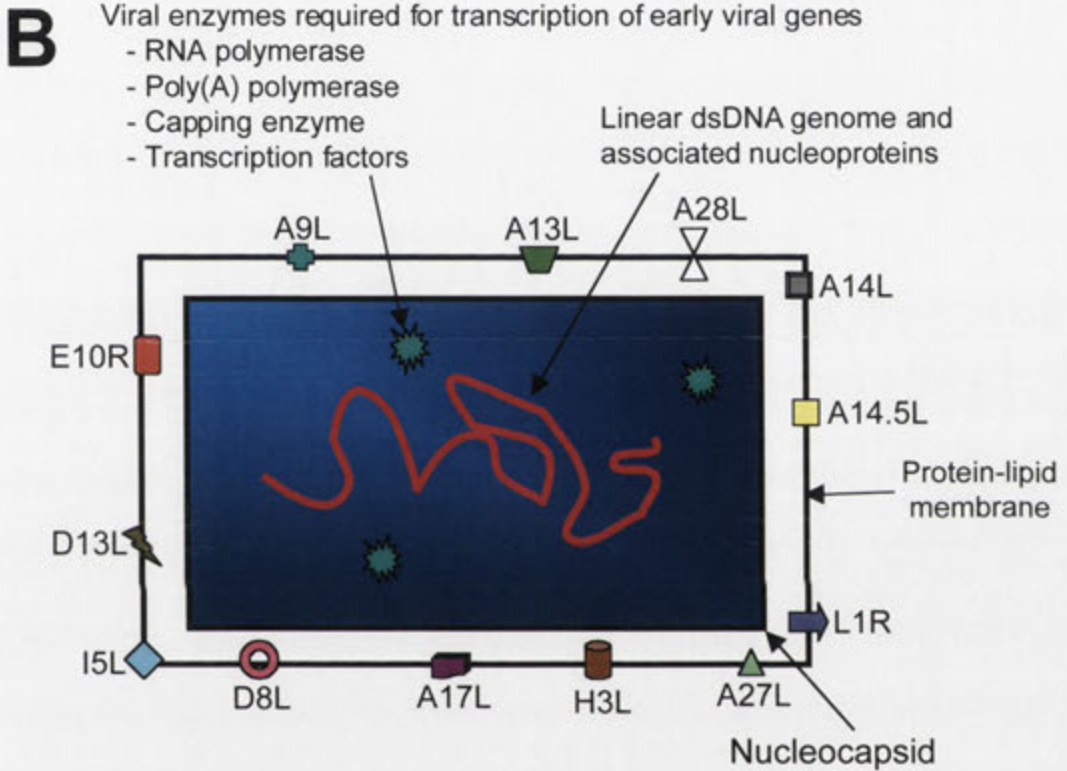
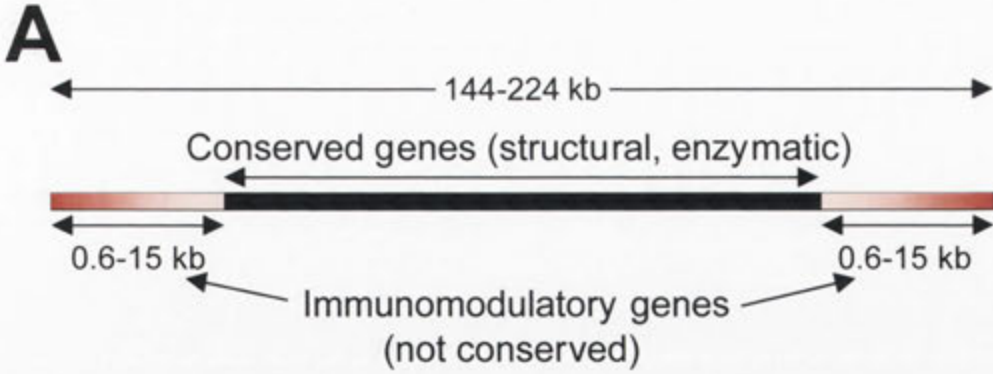
**Table 1.1** – Classification of some important members of the *Chordopoxvirinae* and poxvirus-induced diseases.

Genus	Species	Host	Disease
<i>Orthopoxvirus</i>	Variola virus	Humans	Smallpox
	Vaccinia virus	Humans, mice, rabbits	Cutaneous lesion
	Ectromelia virus	Mice	Mousepox
	Monkeypox virus	Monkeys, humans	Monkeypox
	Camelpox virus	Camels	Camelpox
	Cowpox virus	Rodents, cats, cows, humans	Cutaneous lesion
<i>Parapoxvirus</i>	Orf virus	Sheep, goats	Contagious pustular dermatitis
	Pseudocowpoxvirus	Human	Milker's nodule
		Cattle	Udderpox
	Bovine papular stomatitis virus	Human	Milker's nodule
		Cattle	Bovine papular stomatitis
<i>Capripoxvirus</i>	Goatpox virus	Goats	Goatpox
	Sheeppox virus	Sheep	Sheeppox
	Lumpy skin disease virus	Cattle	Lumpy skin disease
<i>Suipoxvirus</i>	Swinepox virus	Pigs	Swinepox
<i>Avipoxvirus</i>	Fowlpox virus	Chicken	Fowlpox
	Turkeypox virus	Turkey	Turkeypox
	Canarypox virus	Canary	Canarypox
<i>Molluscipoxvirus</i>	Molluscum contagiosum virus	Humans	Molluscum contagiosum
<i>Yatapoxvirus</i>	Tanapox virus	?Rodents, Humans	Cutaneous lesion
	Yaba monkey tumor virus	?Monkeys, Humans	Cutaneous lesion
<i>Leporipoxvirus</i>	Myxoma virus	Tapeti	Cutaneous fibroma
		Brush rabbit	Cutaneous fibroma
		European Rabbit	Myxomatosis
		Eastern cottontail	Cutaneous fibroma
	Rabbit fibroma virus	European Rabbit	Cutaneous fibroma

**Figure 1.1 - Schematic of the poxvirus genome and morphology of vaccinia virus virions.** A: The poxvirus genome is a linear, double-stranded DNA molecule, 144-224 kb in size, with 0.6-15 kb inverted terminal repeats (red) at each end. The central region of the genome is conserved and encodes the structural and enzymatic genes required for virus replication. The inverted terminal repeats are less conserved and primarily encode immunomodulatory genes.

VV particles exist in two infectious forms, intracellular mature virions (IMV; B) and extracellular enveloped virions (EEV; C). IMV particles contain the dsDNA genome of the virus associated with nucleoproteins, and the viral enzymes required for early gene synthesis (such as RNA polymerase, poly(A) polymerase, capping enzyme and transcription factors) inside a protein nucleocapsid. This is then wrapped in a protein-lipid membrane containing 13 viral transmembrane, membrane-bound or membrane-associated antigens (A9L, A13L, A14L, A14.5L, A17L, A27L, A28L, D8L, D13L, E10R, A28L, H3L, I5L and L1R). EEV particles are IMV particles wrapped in another lipid membrane containing 4 glycoprotein antigens on the outer surface (A33R, B5R, A56R and A34R) and one on the inner surface (F13L).





virions (CEV) and play an important role in infecting adjacent cells. The EEV and IMV membranes of vaccinia virus (VV) have different membrane-bound and associated proteins, as shown in Figure 1.1.

### 1.1.2 Poxvirus replication cycle

Poxviruses replicate entirely within the cytoplasm of infected cells (Figure 1.2). To enter the cell, IMV and EEV particles may utilise different receptors, both on the surface of the virus particle and on the host cell (Moss, 2002). The cellular receptors for poxviruses are unknown, and reports suggesting that poxvirus particles bound to the epidermal growth factor receptor (EGFR) (Eppstein *et al.*, 1985) or to chemokine receptors (Lalani *et al.*, 1999b) have not been supported by other groups (Hugin and Hauser, 1994; Masters *et al.*, 2001). VV IMV particles attach to and penetrate the host cell via a number of viral membrane proteins including L1R (Ichihashi *et al.*, 1994; Wolffe *et al.*, 1995), A27L (Rodriguez *et al.*, 1996; Rodriguez *et al.*, 1993; Rodriguez and Esteban, 1987), D8L (Hsiao *et al.*, 1999; Lai *et al.*, 1991a), A28L (Senkevich *et al.*, 2004a; Senkevich *et al.*, 2004b) and H3L (Lin *et al.*, 2000). B5R and A34R may have a role in binding of EEV virions (Galmiche *et al.*, 1999; McIntosh and Smith, 1996). After fusion of the IMV and plasma membranes, the nucleocapsid is released into the cytoplasm. EEV entry is a more complex process due to the extra viral membrane. It is believed that following binding, the EEV is endocytosed, the EEV membrane disrupted due to the low pH of the endosome and the resulting IMV particle fuses with the endosomal membrane to release the viral core into the cytoplasm (Vanderplasschen *et al.*, 1998).

Once inside the cell, the virus core immediately begins transcription of early viral genes using transcription machinery carried within the nucleocapsid. The nucleocapsid is

degraded by a viral protease (Pedley and Cooper, 1987), releasing the viral genomic DNA and early transcripts into the cytoplasm. Early viral genes encode the proteins required for viral DNA replication and the expression of intermediate viral genes, as well as those responsible for immune modulation and suppression. The immunomodulatory genes of myxoma virus will be discussed in detail in Section 1.2.4. The poxvirus genome is replicated entirely within the cytoplasm in discrete regions known as factory areas or viriosomes (Moss, 2002). The genome is replicated as concatemers and resolved into individual genomic units, in a process not fully understood (Moss, 2002).

Expression of intermediate and late genes follow DNA replication (Moss, 1990). Late genes include the structural proteins, which go on to make progeny virus as well as those required for assembly of new virus particles. Virus assembly occurs at discrete cytoplasmic sites visible as membrane crescents, which go on to form the IMV membrane. These membranes become spherical, enclosing viral nucleoprotein, DNA and all of the required structural proteins and enzymes. This structure eventually matures into an infectious IMV particle. These can then be transported along microtubule networks to regions where they are wrapped in two more lipid membranes derived from the trans-Golgi or endosomal network (Smith *et al.*, 2002), to form intracellular enveloped virions (IEV). These particles are moved on actin tails that form on viral protein complexes on the outer surface of IEV particles, which move them to the plasma membrane. Fusion of the outer IEV membrane with the plasma membrane releases a mature EEV particle, which can be forced away from the cell on the actin tail or enter adjacent cells (Smith *et al.*, 2002).

## 1.2 MYXOMA VIRUS AND MYXOMATOSIS

Myxoma virus is the prototype member of the *Leporipoxvirus* genus, which also includes the antigenically related rabbit (or Shope) fibroma virus (RFV), squirrel fibroma virus and hare fibroma virus (Esposito and Fenner, 2002; Moss, 2002). Myxoma virus infects members of the family *Leporidae* including rabbits and hares. The virus released in Australia and Europe originated in the South American jungle rabbit, also known as the tapeti (*Sylvilagus brasiliensis*), in which it induces a benign cutaneous lesion at the site of inoculation. However, in the European rabbit (*Oryctolagus cuniculus*), the virus causes myxomatosis, a systemic disease characterised by large mucoid cutaneous swellings (myxomas) with virus replication occurring in many tissues within the host, and which is highly lethal in susceptible rabbits (Kerr and McFadden, 2002). There are also strains of myxoma virus which are found in the Californian brush rabbit, *Sylvilagus bachmani*, and referred to as Californian myxoma virus. These strains are highly lethal in domestic European rabbits, more so than South American strains, but do not induce florid clinical myxomatosis before death except in occasional rabbits which survive for more than 12 days (Fenner and Ratcliffe, 1965).

### 1.2.1 History of myxomatosis in Australian wild rabbits

Wild European rabbits were first successfully to mainland Australia in 1859 near Geelong. Over the next 60 years, rabbits rapidly spread over most of the continent, causing extensive environmental and agricultural damage (Fenner and Ratcliffe, 1965; Myers *et al.*, 1994). The use of myxomatosis as a biological control against rabbits was suggested in 1919 (Fenner and Ratcliffe, 1965). This was followed by experimental trials in the 1930's and 40's. However, successful spread of the virus did not occur until summer 1950 following experimental release at Gunbower, Victoria (Fenner and Ratcliffe, 1965). The virus rapidly

spread throughout the Murray-Darling basin over the next few months, and to the rest of the continent following a widespread inoculation campaign. The originally released virus (the Standard Laboratory Strain, SLS) was 99.8% lethal in infected rabbits (Fenner and Ratcliffe, 1965). The rabbit population dropped significantly during the original epizootics, reducing rabbit numbers to about 5% of the pre-myxoma virus levels (Williams *et al.*, 1995). Myxoma virus was later deliberately released into France where it spread to the rest of Europe and the United Kingdom (Fenner and Ratcliffe, 1965).

Since the release of myxoma virus in Australia and Europe, the virus and rabbit have co-evolved. Attenuated strains of the virus have become dominant in the field, and wild rabbit populations have developed resistance to the virus (reviewed in detail in Fenner and Ratcliffe, 1965; Kerr and Best, 1998). Moderately attenuated strains of the virus transmitted more effectively than the highly virulent SLS strain due to longer survival times of infected animals, allowing a greater period of time during which the virus could be transmitted by insect vectors. The emergence of attenuated viruses may have allowed for the survival and reproduction of genetically resistant rabbits. Myxoma virus is now endemic in wild rabbit populations and is still responsible for killing 50-60% of infected wild rabbits in Australia (Kerr and Best, 1998).

### **1.2.2 Domestic rabbits and myxomatosis in Australia**

Since the release of rabbit haemorrhagic disease virus (RHDV) in Australia in 1996, wild rabbit numbers have dropped significantly in many parts of the country (Kovaliski, 1998; Mutze *et al.*, 2002), including areas in which wild rabbit harvesting operations for meat and fur was an important industry. Since this time, the raising of rabbits as farmed animals for meat and fur production has been legalised by most State governments, and a shift toward

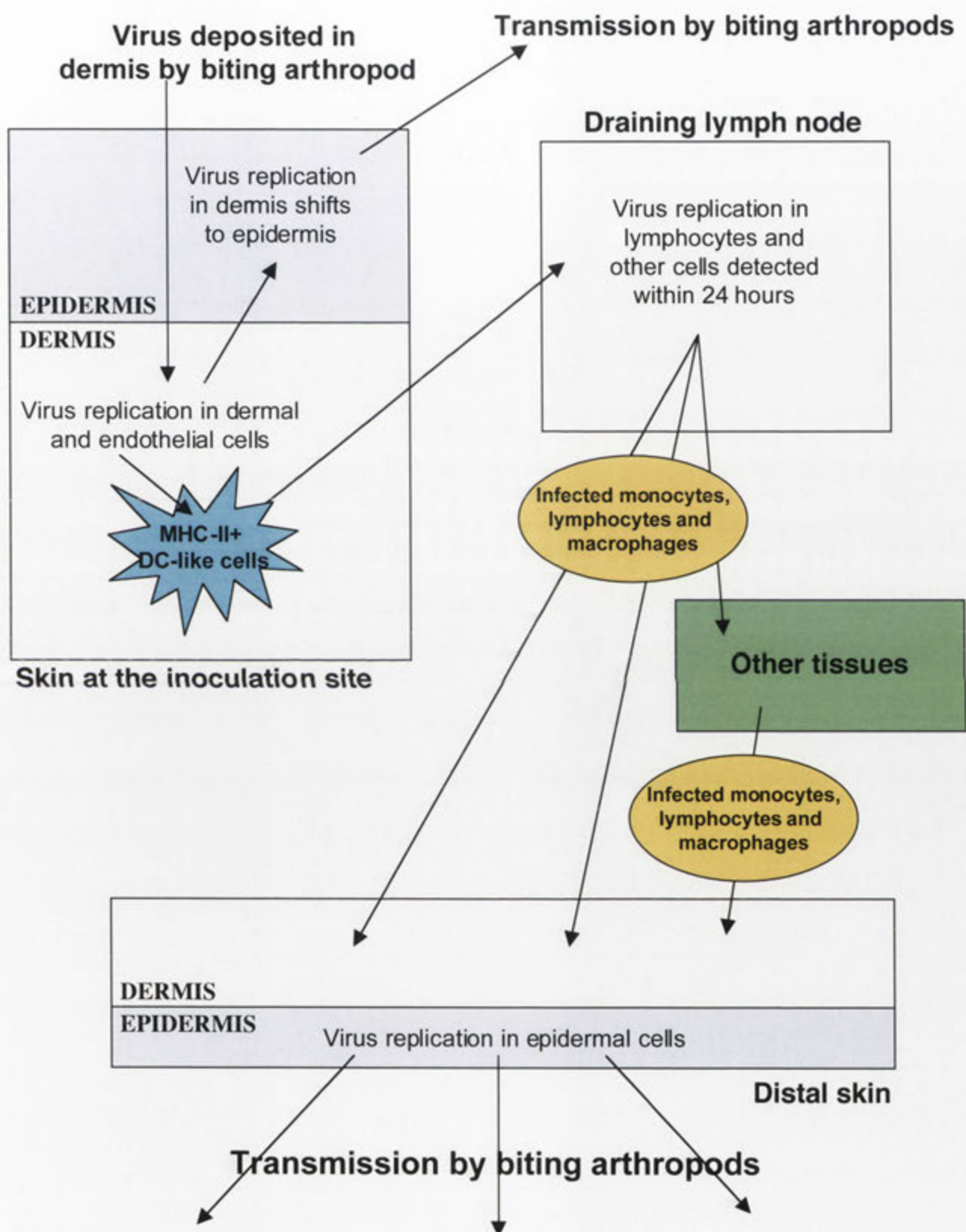
domestic rabbit farming from wild rabbit harvesting has occurred. In 1999, the Australian farmed rabbit industry was estimated to be worth A\$620,000 with an annual domestic growth rate of 31% (Foster, 1999).

Myxomatosis has the potential to cause devastating losses to rabbit farms following transmission to domestic populations from wild rabbit populations where the virus is endemic (Frolich *et al.*, 2002). Moreover, myxomatosis vaccines are not permitted in Australia due to fears that vaccine virus could be transmitted from recently vaccinated animals to wild rabbit populations (Fenner and Ross, 1994). There are fears that a live vaccine virus could become established amongst wild rabbits and reduce the efficacy of myxoma virus as a population control (Fenner and Ross, 1994). In the absence of a vaccine, the only control mechanisms for myxomatosis are to exclude insect vectors by maintaining insect proof facilities, and through the culling of infected rabbits (Foster, 1999).

### **1.2.3 Pathogenesis of myxoma virus in domestic European rabbits**

The pathogenesis of virulent and attenuated strains of myxoma virus in susceptible (domestic) and genetically resistant (wild) rabbits has been previously studied (Best *et al.*, 2000; Best and Kerr, 2000). The current model for myxoma virus pathogenesis is displayed in Figure 1.3 and is based on the following experimental data. After inoculation of the virus into the epidermis by an arthropod vector, the virus initially replicated in MHC-II<sup>+</sup> dendritic-like cells in the dermis and in endothelial cells (Best *et al.*, 2000). Dramatic histological changes were observed in the skin, both at the primary inoculation site and secondary skin lesions. There was proliferation of the epithelial cells, and associated thickening of the epidermis, followed by degradation of the collagen structure in the dermis

**Figure 1.3 - Pathogenesis of myxoma virus in European rabbits.** The virus is deposited in the skin by biting insects, and replication is first seen in MHC-II+ dendritic-like cells (DC) in the dermis. Within 24 hours the virus reaches the draining lymph node where it replicates within lymphocytes within the T cell zone. The virus disseminates in infected lymphocytes, monocytes and macrophages to other tissues including the spleen, testis, lung, distal skin and mucocutaneous sites including the nose and conjunctivae. At the inoculation site and distal skin sites, virus replication shifts to the epidermal layer, where it replicates to high titres, and can be transmitted by biting insects. Figure adapted from Kerr and McFadden (2002).





and epidermis, vesicle formation and scabbing (Hurst, 1937). A limited inflammatory response was seen in the skin, usually taking the form of an influx of polymorphonuclear cells into the dermis and subdermis. Endothelial cells surrounding the blood vessels of the epidermis and dermis proliferated, and large stellate cells called myxoma cells were often associated with small blood vessels, and believed to be derived from infected endothelial cells (Best *et al.*, 2000; Hurst, 1937).

Within 24 hours, virus was detected in the draining lymph node, replicating in the T cell zone. Infection of lymphocytes resulted in an extensive reduction in the lymphocyte population initially within the paracortex, and later in the follicles (Best *et al.*, 2000). The virus disseminated to other lymphoid tissues (distal lymph nodes, spleen), the lungs, testes, secondary skin sites and mucocutaneous surfaces of the eyelids and nose. As virus was not detectable as free virions in the serum, it is believed that it disseminated to distal sites within infected leukocytes (Fenner and Woodroffe, 1953). The infection and widespread dysfunction of these cells are believed to play a key role in the development of systemic immune suppression and poor innate and adaptive immune responses to the virus and secondary infections. The virus can be transmitted to other animals from primary and secondary skin lesions by biting arthropods due to high titres of virus in the epidermis (Fenner and Ratcliffe, 1965; Joubert *et al.*, 1967; Mead-Briggs and Vaughan, 1975; Shepherd and Edmonds, 1977). The virus does not replicate in the insect vector, but is passively transferred on the mouthparts of the insect (Day *et al.*, 1956).

#### *1.2.3.1 Clinical manifestations of myxomatosis*

The first clinical sign of infection is a lesion developing at the cutaneous inoculation site within 2-4 days post inoculation. This lesion begins as a small red swelling, grows up to 60

mm in diameter and can be highly protuberant (20 mm high) depending on the strain of virus (Fenner and Ratcliffe, 1965). Between 6 and 8 days, secondary signs of myxomatosis can be observed as swellings on the mucocutaneous surfaces of the conjunctivae and nose that develop into distinct secondary lesions. The eyelids can swell considerably, and secondary bacterial infections result in mucopurulent discharge that can completely block the eyes. Mucopurulent discharge in the nasal passages can obstruct the airways resulting in laboured, 'snuffly' breathing. Secondary skin lesions develop over the entire body, but are most prominent on the face, ears and eyelids. In rabbits that survive infection, the clearance of these secondary lesions can result in scarring, loss of fur and a 'moth-eaten' appearance, most obvious on the thin tissue of the ears, which is a major concern to owners of show animals and pets (NSW Rabbit Fanciers Association, personal communication).

In male rabbits, the virus replicates to high titres in the testes resulting in oedematous swelling of the scrotum, an acute inflammatory response in the interstitium, necrosis of tubular cells and epididymitis (Fenner and Woodroffe, 1953; Hurst, 1937). Infection with attenuated strains of myxoma virus can result in long term infertility in male rabbits (Fountain *et al.*, 1997; Sobey and Turnbull, 1956), or transmission of the virus in semen during artificial insemination (Marlier *et al.*, 2000), both major concerns for rabbit breeders.

The cause of death in myxoma virus-infected rabbits is unclear. There is no obvious pathology in any key organ in which virus can be detected that would be expected to lead to death (Mims, 1964). Generalised immunosuppression caused by the virus may allow secondary bacterial infections to occur in the upper respiratory tract, with resultant respiratory difficulties. This has been considered the primary cause of fatality through

obstruction of the respiratory tract by mucopurulent discharge (Hobbs, 1928), however in acute infections, rabbits succumb to the virus with little pathology in the lungs or evidence of significant secondary infections (Fenner and Ratcliffe, 1965), indicating that there are other causes of death.

#### *1.2.3.2 Genetic resistance of Australian wild rabbits*

The mechanisms underlying the genetic resistance of wild rabbits have not been characterised and are currently under investigation (Best *et al.*, 2000; Best and Kerr, 2000; Kerr and McFadden, 2002; Kerr *et al.*, 2004). In genetically resistant rabbits, the extent of pathology in the draining lymph node observed 4 days post inoculation was reduced compared to susceptible rabbits, and there was less virus detected at distal tissues (Best and Kerr, 2000). It appears that resistant rabbits are able to control the replication of myxoma virus early in infection through enhanced innate immune mechanisms, before generalised virus-induced immunosuppression sets in. This allows them to develop a virus-specific cell-mediated response sufficient to control and clear the virus (Best *et al.*, 2000; Best and Kerr, 2000).

#### **1.2.4 Immunomodulatory proteins of myxoma virus**

Of the 156 predicted open reading frames in myxoma virus, 31 have demonstrated or predicted immunoregulatory or host range functions (Barrett *et al.*, 2001; Cameron *et al.*, 1999). Many of these have been intensively studied both *in vivo* and *in vitro* as a model system for poxvirus immunomodulatory strategies and have been reviewed in detail (Nash *et al.*, 1999; Zuniga, 2002).

Poxvirus immunomodulatory proteins have distinct functions and benefits for the virus (Table 1.2). These can be classed into three broad groups including viroceptors, which are secreted from infected cells and mimic host cellular receptors, binding to and neutralising specific immune modulators (M-T1, M-T2, M-T7); virokines, which are secreted from infected cells and mimic the structure of host cytokines, immune system inhibitors or growth factors (MGF); and viromitigators, intracellular proteins which play a role in virus host range (both species host range and cell types within the host) and regulation of apoptosis (M-T4, M-T5, M011L, M029R, M063R, M151R, M153R and M152R).

During the course of this project, four live, attenuated myxoma virus vaccine candidates were constructed by inactivating key viral immunomodulatory and host range genes. The functions of myxoma virus immunomodulatory proteins that are of importance to this project (M-T7, M010L, M011L and M063R) are described in detail below.

#### *1.2.4.1 The interferon- $\gamma$ binding protein – M-T7*

All Chordopoxviruses encode an interferon- $\gamma$  (IFN- $\gamma$ ) binding protein (Smith *et al.*, 1998). Inhibition of IFN- $\gamma$  action is advantageous to poxviruses due to the central role of the cytokine in innate and cell-mediated adaptive immune responses (Boehm *et al.*, 1997). IFN- $\gamma$  is a pleiotropic cytokine secreted by activated T cells and NK cells. It is active as a 34 kDa homodimer, and binds to a specific IFN- $\gamma$  receptor ubiquitously expressed on all nucleated cells, leading to the phosphorylation of STAT1 $\alpha$  transcriptionally active subunits and the transcription of specific interferon response genes (Boehm *et al.*, 1997). IFN- $\gamma$  has many effects on host cells including the upregulation of MHC-I and MHC-II expression, the promotion of Th1 differentiation and inhibition of Th2 differentiation of CD4<sup>+</sup> T helper cells, the activation of macrophages and the induction of inducible nitric oxide synthetase

**Table 1.2** – Selected immunomodulatory proteins of myxoma virus.

Open Reading Frame	Size (aa)	Function	Virulence of knockout virus	References
M001R/L	260	Secreted chemokine binding protein (MT1)	virulent	Graham <i>et al.</i> (1997) Lalani <i>et al.</i> (1998) Lalani <i>et al.</i> (1999a) Seet <i>et al.</i> (2001)
M002R/L	326	Soluble TNF receptor homologue/apoptosis regulator (MT2). Intracellular and secreted	attenuated	Schreiber <i>et al.</i> (1996) Schreiber and McFadden (1996) Schreiber <i>et al.</i> (1997)
M004R/L	237	ER localised apoptosis regulator (MT4)	attenuated	Barry <i>et al.</i> (1997) Hnatiuk <i>et al.</i> (1999)
M005R/L	483	Cytoplasmic apoptosis regulator	attenuated	Mossman <i>et al.</i> (1996a)
M007R/L	263	Secreted $\gamma$ -interferon receptor homologue (MT7); chemokine binding protein	attenuated	Lalani <i>et al.</i> (1997) Mossman <i>et al.</i> (1995b) Upton <i>et al.</i> (1992) Mossman <i>et al.</i> (1996b)
M008.1R/L	369	Secreted anti-inflammatory serpin (Serp 1)	attenuated	Macen <i>et al.</i> (1993) Upton <i>et al.</i> (1990) Nash <i>et al.</i> (1998) Hatton <i>et al.</i> (2000)
M010L	85	EGF-like growth factor (MGF)	attenuated	Opgenorth <i>et al.</i> (1992) Lin <i>et al.</i> (1991) Opgenorth <i>et al.</i> (1993)
M011L	166	Anti-apoptotic integral mitochondrial membrane protein	attenuated	Everett <i>et al.</i> (2000) Macen <i>et al.</i> (1996) Opgenorth <i>et al.</i> (1992)
M151R	326	Cytoplasmic serpin (Serp 2)	attenuated	Messud-Petit <i>et al.</i> (1998) Turner <i>et al.</i> (1999)
M152R	266	Cytoplasmic serpin (Serp 3)	attenuated	Guerin <i>et al.</i> (2001)
M153R	206	Myxoma virus leukemia-associated protein, MHC-I down-regulation	attenuated	Guerin <i>et al.</i> (2002)

Table 1.2 continued

M029L	115	PKR inhibition, interferon resistance/host range. Cytoplasmic	N/D*	Uncharacterised in myxoma virus
M062R	158	Virulence factor/host range	N/D*	Uncharacterised in myxoma virus
M063R	215	FAS binding death associated protein domain/apoptosis regulator/interferon resistance	Attenuated	Grant McFadden, personal communication
M064R	203	Virulence factor/host range. Cytoplasmic	N/D*	Uncharacterised in myxoma virus
M156R	102	Structural mimic of eIF2 $\alpha$ ; cytoplasmic; inhibition of PKR; interferon resistance	N/D*	Ramelot <i>et al.</i> (2002)

\* - Not done

(iNOS) (Boehm *et al.*, 1997; Goodburn *et al.*, 2000; Revel and Chebath, 1986). IFN- $\gamma$  also establishes an ‘antiviral’ state within cells through the transcriptional induction of dsRNA-activated protein kinase (PKR) (Hovanessian, 1993), 2’-5’ oligoadenylate synthetase (2-5A synthetase) (Graham *et al.*, 1993) and dsRNA-specific adenosine deaminase (dsRAD) (Patterson *et al.*, 1995). These proteins recognise and are activated by dsRNA molecules produced during viral infection, and lead to the inhibition of protein synthesis and non-specific degradation of mRNA, inhibiting viral replication (Boehm *et al.*, 1997; Goodburn *et al.*, 2000). Mice with deleted IFN- $\gamma$  or IFN- $\gamma$  receptor genes show increased susceptibility to many pathogens including the poxviruses VV and ectromelia virus (Huang *et al.*, 1993; Karupiah *et al.*, 1993; Muller *et al.*, 1994).

The first viral IFN- $\gamma$  receptor homologue was discovered in myxoma virus as a secreted protein with a high degree of homology to the extracellular domains of the cellular IFN- $\gamma$  receptor (Upton *et al.*, 1992). This protein (M-T7) is encoded by the gene M007, which is present as two copies, one in each of the inverted terminal repeats of the myxoma virus genome. M-T7 is a 37 kDa glycoprotein and is the major protein secreted from cells infected with myxoma virus (Upton *et al.*, 1992). It specifically binds rabbit IFN- $\gamma$  and prevents its binding to cellular IFN- $\gamma$  receptors (Mossman *et al.*, 1995b).

M-T7 is a key virulence factor of myxoma virus (Mossman *et al.*, 1996b). Disruption of both M007 reading frames resulted in a highly attenuated virus with mortality in domestic rabbits reduced to 8% compared to 100% for wild type virus (Mossman *et al.*, 1996b). The infection was characterised by reduced symptoms of disease including fewer and less severe secondary lesions. Lesions were characterised by a massive increase in cellular infiltrate into the sites of infection, including active lymphocytes and macrophages

(Mossman *et al.*, 1996b). The effects of M-T7 deletion were systemic, in that secondary lymphoid organs such lymph nodes and spleens contained much greater levels of reactive lymphocytes and a generally more vigorous cellular immune response compared to wild type myxoma virus-infected rabbits (Mossman *et al.*, 1996b).

M-T7 also binds to a range of host C, CC and CXC chemokines through their heparin-binding domains (Lalani *et al.*, 1997). Chemokines are a large family of small proteins which form the communication system of the immune system (Laing and Secombes, 2004; Rot and von Andrian, 2004). They are released at sites of infection or by specific immune cells and establish chemoattractant gradients used by leukocytes to migrate to the appropriate regions. The release of M-T7 by virus-infected cells has a dramatic effect on leukocyte migration in myxoma virus-infected tissues, preventing the influx of lymphocytes to the site of infection (Lalani *et al.*, 1997; Mossman *et al.*, 1996b). Myxoma virus also encodes M-T1, which binds to CC chemokines (Lalani *et al.*, 1999a; Seet *et al.*, 2001), and two uncharacterised proteins (M144R, M118L) with postulated chemokine-modulating properties (Barrett *et al.*, 2001; Cao and McFadden, 2001). The overall result of M-T7 secretion from infected cells is an abrogation of innate antiviral responses induced by IFN- $\gamma$ , and the disruption of chemokine gradients, inhibiting the directional infiltration of leukocytes (Lalani *et al.*, 1997; Mossman *et al.*, 1996b).

#### 1.2.4.2 Myxoma growth factor – M010L

The myxoma growth factor (MGF) is an 85 amino acid (aa) polypeptide encoded by the gene M010L (Lin *et al.*, 1991). This highly glycosylated protein is secreted early during infection and plays a role in the proliferation of cells at primary and secondary sites of infection including the skin, conjunctiva and respiratory tract (Opgenorth *et al.*, 1992). It



has been postulated that the mitogenic action of EGF homologues encoded by poxviruses stimulate higher levels of biosynthetic activity in infected and adjacent cells, providing a local environment allowing for enhanced replication of the virus (McFadden *et al.*, 1995).

Deletion of MGF from virulent strains of myxoma virus results in attenuation of the infection (Opgenorth *et al.*, 1992). Secondary lesions on the body and conjunctiva were observed, but the disease was milder than in rabbits infected with wild type myxoma virus, and resulted in only 25% mortality in domestic rabbits. Epithelial cell layers over the primary lesion and secondary lesions of the conjunctiva and lungs contained fewer proliferating cells. Attenuation was suggested to be due to less proliferation-induced damage to epithelial cell layers at sites of infection, especially in the lungs, resulting in a reduced level of bacterial infections (Opgenorth *et al.*, 1992).

#### 1.2.4.3 M011L

M011L encodes a 166 aa protein that plays an important role in myxoma virus pathogenesis. The protein is synthesised early in infection and possesses a C-terminal hydrophobic helix and an N-terminal signal sequence which localises the protein to the outer mitochondrial membrane (Everett *et al.*, 2000; Graham *et al.*, 1992). Domestic rabbits infected with a strain of myxoma virus with an inactivated M011L open reading frame ( $\Delta$ M011L) survived infection with only mild signs of myxomatosis (Opgenorth *et al.*, 1992). Lesions were characterised by a more intense inflammatory response with an increased polymorphonuclear cell infiltrate (Opgenorth *et al.*, 1992). There was also less lymphocyte depletion in the spleen (Opgenorth *et al.*, 1992).

*In vitro*, ΔM011L viruses exhibited normal growth characteristics in rabbit fibroblast cell lines. However, in the rabbit CD4<sup>+</sup> T lymphocyte cell line RL-5, apoptosis was induced resulting in a non-productive infection of these cells (Macen *et al.*, 1996). M011L expression was also required to inhibit apoptotic responses in infected monocytes and macrophages (Everett *et al.*, 2000), and its anti-inflammatory properties were mediated by the prevention of inflammatory responses induced by apoptotic monocytes (Savill, 1997). M011L prevented the loss of mitochondrial membrane potential which otherwise led to the activation of caspase-9 and the apoptosis of infected cells (Everett *et al.*, 2002; Saelens *et al.*, 2004).

#### 1.2.4.4 Host range genes - intracellular inhibitors of interferons

A host range protein is one that allows the infecting virus to productively infect a cell. The deletion of any single host range protein usually has lethal effects on poxvirus replication in many cell lines, resulting in a non-productive infection, and/or attenuation of the virus *in vivo*. Poxvirus host range genes (viromitigators) can be classified into two groups. The first are proteins that interrupt specific cellular pathways leading to apoptosis. In myxoma virus, these include M-T2, M-T4, M-T5, M011L, M143R and M151R (Barrett *et al.*, 2001). The second are proteins that play a role in preventing the action of interferon within infected cells, through the interruption of pathways and intermediates that lead to the activation of interferon response genes (Smith *et al.*, 1998).

During this project, a myxoma virus vaccine that was replication-deficient in rabbit cells was constructed by deleting M063R, a gene that plays an unidentified role in the interruption of host cell responses to interferons (Grant McFadden, personal communication). M063R belongs to a class of poxvirus proteins containing the conserved

Pox\_C7\_F8A domain. There are 24 proteins containing this motif amongst the Chordopoxviruses, including 3 myxoma virus proteins (M062R, M063R and M064R) (Cameron *et al.*, 1999), the VV protein C7L (Oguiura *et al.*, 1993) and the capripoxvirus protein CF8A (Gershon and Black, 1989). Deletion of C7L from the genome of VV resulted in a virus with reduced replication ability in hamster Dede cells, but normal replication in most human cell lines (Oguiura *et al.*, 1993). The myxoma virus genes homologous to C7L and their mechanism of action have not been fully characterised, although are believed to be through the impediment of the infected cell's response to interferons (Grant McFadden, personal communication). Deletion of M063R from the genome of myxoma virus resulted in a virus unable to replicate in rabbit cells, but able to replicate in several primate cell lines (Grant McFadden, personal communication).

Several other poxvirus proteins have well-defined roles in inhibiting host cell responses to interferons. M156R is a structural mimic of the eukaryotic translation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) (Ramelot *et al.*, 2002). M156R competes with eIF2 $\alpha$  as a substrate for phosphorylation by the interferon-inducible protein kinase PKR, thereby preventing the phosphorylation of eIF2 $\alpha$  and the resultant inhibition of translation. Homologues of M156R are present in many other poxviruses including the well-characterised VV protein K3L (Beattie *et al.*, 1991; Carroll *et al.*, 1993; Davies *et al.*, 1992; Kawagishi-Kobayashi *et al.*, 1997) and swinepox C8L (Kawagishi-Kobayashi *et al.*, 2000).

M029R is a homologue of the VV protein E3L (Barrett *et al.*, 2001), a well-characterised protein that prevents the activation of IFN-activated genes. E3L has been shown to bind to dsRNA produced during viral infection and thereby preventing the activation of PKR (and protein translation inhibition through eIF2 $\alpha$ ) and 2-5A synthetase (and the subsequent

activation of RNaseL) (Garcia *et al.*, 2002; Langland and Jacobs, 2002; Rivas *et al.*, 1998; Sharp *et al.*, 1998; Smith *et al.*, 2001; Xiang *et al.*, 2002). Together, E3L and K3L directly block the intracellular antiviral activity of interferons, preventing the interferon-mediated cessation of biosynthetic activity within VV-infected cells.

### **1.3 IMMUNE RESPONSES TO POXVIRUS INFECTIONS**

When considering vaccine design, it is imperative to distinguish between immune responses that are responsible for *clearance of an infection*, and the long-term memory responses that are critical for *protection from further challenge*. Knowledge of the virus-specific memory responses required for long-term protection are essential to the rational design of a poxvirus vaccine that must induce these responses. However, consideration of virus clearance is also important as the majority of poxvirus vaccines are live viruses that must be cleared from the vaccinated host, and in the process induce virus-specific circulating antibody and memory T and B cells that can confer the desired level of protection from challenge.

#### **1.3.1 Poxvirus disease models - why use the myxoma virus/rabbit model?**

The majority of research into the immunological responses to poxvirus infection has been undertaken in two mouse models - vaccinia virus (VV) and ectromelia virus (EV). The diversity of immunological tools available for mice has resulted in the elegant dissection of the specific immune responses involved in virus clearance and protection. The EV/mouse model is a natural poxvirus model, and the virus is a naturally transmitted pathogen (Turner and Moyer, 2002). A low dose of virus (<10 pfu) delivered subcutaneously can replicate, disseminate and induce a fatal systemic disease (Fenner, 1981). EV has been used to determine the host immune responses critical for control, clearance and recovery from a

virulent poxvirus infection (Atrasheuskaya *et al.*, 2004; Chaudhri *et al.*, 2004; Karupiah, 1998; Karupiah *et al.*, 1996; Karupiah *et al.*, 1993). However, the EV/mouse model has not been used to elucidate the specific immune responses required for protection from re-challenge.

The VV/mouse model has been used extensively in recent years to determine the host factors that can confer immunity to challenge with poxviruses (Belyakov *et al.*, 2003; Wyatt *et al.*, 2004; Xu *et al.*, 2004). However, it is important to note that the VV/mouse model is not a natural disease model, and the results obtained cannot be directly extrapolated to natural poxvirus disease models (Turner and Moyer, 2002). VV is only lethal in mice at very high doses ( $>10^6$  pfu) delivered by intracranial, intranasal or intraperitoneal routes. Intraperitoneal challenge is an artificial mode of infection as the virus is immediately disseminated to all internal organs, and intranasal infection is only lethal with certain strains of VV such as Western Reserve (WR), and lethality is dose-dependant (Turner, 1967). 'Natural' infection via the skin results in a non-disseminating, non-lethal disease in immunocompetent mice (Tscharke and Smith, 1999; Turner, 1967). The deletion of many key VV genes only alters the pathogenicity of VV in mice during intradermal infection, not intranasal challenge (Tscharke *et al.*, 2002). Other key immunomodulatory proteins do not affect VV virulence as they do not interact with murine substrates. For instance, the VV IFN- $\gamma$ -binding protein B8R binds to murine IFN- $\gamma$  with a much lower affinity than to IFN- $\gamma$  of other species, and deletion of B8R does not alter the pathogenesis of intranasal VV infection in mice (Mossman *et al.*, 1995a; Symons *et al.*, 2002).

Outside of the Orthopoxviruses, there is no mouse model for poxvirus diseases. The lack of immunological tools and in-bred strains available for non-mouse species makes it difficult to elucidate the immune mechanisms that are responsible for poxvirus clearance and protection.

This study has utilised the rabbit/myxoma virus model to evaluate the protective efficacy of various vaccination strategies in a natural disease model. This model utilises a laboratory animal and a well-characterised virus with defined pathogenesis. Infection with a small, naturally delivered (intradermal inoculation) dose of virulent virus results in dissemination of the virus and a 100% lethal disease. The protective efficacy of vaccines in this model can be extrapolated to other natural poxvirus disease models including capripoxvirus infections and smallpox because a vaccine design that protects rabbits from myxomatosis is likely provide a model for vaccines to protect other hosts from diseases with a similar disseminating, lethal pathology.

### **1.3.2 Immune mechanisms responsible for clearance of poxvirus infections**

#### *1.3.2.1 Antibody*

Anti-poxvirus antibody does not appear to play an important role in the clearance of, and survival from, many primary poxvirus infections, although anti-virus antibody develops quickly following infection. Antibody is detected as soon as 6 days after infection in VV-infected mice (Hutt, 1975; Novembre *et al.*, 1989) and myxoma virus-infected rabbits (Best and Kerr, 2000), and by 7 days post infection in EV-infected mice (Chaudhri *et al.*, 2004).

In VV-infected mice, cell-mediated immune mechanisms (cytotoxic T lymphocytes [CTL] and natural killer [NK] cells) peak and contract before significant neutralising antibodies

are detected, suggesting that antibodies play a relatively minor role in clearance of the primary VV infection (Novembre *et al.*, 1989). This is further supported by the fact that humans with Burton's agammaglobulinemia, a genetic defect that renders them incapable of generating antibody, generally do not display adverse reactions to VV vaccination and are still capable of developing protective immunity to smallpox in the complete absence of antibody (Fulginiti *et al.*, 1968; Kempe, 1960).

However, other studies have suggested that antibodies are required in the clearance of poxviruses. IgH<sup>-/-</sup> (antibody-deficient) mice infected with VV retained higher titres of VV at 14 dpi than wild type mice did (Xu *et al.*, 2004). Similarly, B cell deficient mice develop a persistent EV infection despite the generation of potent anti-EV cell-mediated immunity (Karupiah, 1998). The infection is resolved following transfer of immune serum or naïve B cells (Karupiah, 1998).

#### *1.3.2.2 Cell-mediated immune responses - cytotoxic T lymphocytes (CD8<sup>+</sup> T cells) and helper T cells (CD4<sup>+</sup> T cells)*

Infection with poxviruses induces high levels of virus-specific T lymphocytes which are principally responsible for clearance of the primary infection (Blanden, 1974; Buller and Palumbo, 1991). Virus-specific T cell responses have been identified in mice infected with EV (Karupiah *et al.*, 1996) or VV (Demkowicz *et al.*, 1992; Xu *et al.*, 2004), humans infected with VV (Demkowicz and Ennis, 1993; Demkowicz *et al.*, 1996; Hammarlund *et al.*, 2003), sheep infected with VV (Issekutz, 1984; Issekutz, 1985), pigs infected with swinepox virus (Williams *et al.*, 1989), and chickens infected with fowlpox virus (Singh and Tripathy, 2003). The anti-poxviral activity of virus-specific T cells *in vivo* is primarily through their direct cytotoxic action on infected cells, and through the antiviral activity of

IFN- $\gamma$  secreted from active CD8<sup>+</sup> and CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells also have a role in the development of humoral responses in mice infected with Orthopoxviruses (Belyakov *et al.*, 2003; Ennis *et al.*, 2002; Karupiah, 1998; Karupiah *et al.*, 1996; Karupiah *et al.*, 1993; Mullbacher, 2003).

The critical importance of CD8<sup>+</sup> T cells in the recovery of mice infected with EV has been well established (Blanden, 1970; O'Neill and Blanden, 1983; O'Neill and Brennan, 1987). Depletion of all T cells prevented the recovery of EV-infected mice (Blanden, 1970; Blanden, 1971; Blanden, 1974). C57BL/6 mice depleted of, or lacking, CD8<sup>+</sup> T cells succumbed to EV with significantly higher titres of virus detected in the spleen and liver than in normal mice (Karupiah *et al.*, 1996). Mice depleted of CD4<sup>+</sup> cells did not succumb to infection, although they had higher virus titres, lower virus-specific CTL activity and became persistently infected with virus (Karupiah *et al.*, 1996).

The relative importance of CD8<sup>+</sup> and CD4<sup>+</sup> T cells appears to be reversed in VV-infected mice. CD8 knockout mice were able to overcome even high doses of intradermally delivered VV with little pathology (Spriggs *et al.*, 1992). Similarly, CD8<sup>+</sup> T cell-depleted mice challenged intraperitoneally with VV showed a similar pathology to VV-infected control mice (Xu *et al.*, 2004). However, depletion of CD4<sup>+</sup> T cells before VV challenge prevented mice from clearing VV effectively, with reduced virus-specific CTL and antibody responses (Xu *et al.*, 2004). In a separate non-lethal VV infection study, it was found that compared to untreated mice, CD4<sup>+</sup> T cell-depleted mice displayed normal pathology, CD8<sup>+</sup> T cell depleted mice had slightly increased weight loss and mice with both CD4<sup>+</sup> and CD8<sup>+</sup> T cells depleted succumbed to the infection (Belyakov *et al.*, 2003). Although CD4<sup>+</sup>-deficient mice appear to mount normal CD8<sup>+</sup> CTL responses, the



importance of CD4<sup>+</sup> T cells in recovery seems to be their crucial role in the development of early antibody responses (Karupiah *et al.*, 1996; Mullbacher, 2003).

There is little information on the importance of cell-mediated immune responses outside of inbred mouse model studies. However, the importance of cell-mediated immunity in the clearance of VV in humans has been well-documented, as it was found that individuals with deficient T lymphocyte responses were more prone to develop disseminated VV disease following vaccination, indicating poor control of the VV infection (Fenner, 1993; Fulginiti *et al.*, 1968; O'Connell *et al.*, 1964).

#### *1.3.2.3 Cytokines in the clearance of poxvirus infections*

The importance of Th1 responses, and especially Th1 cytokines, in the control and clearance of poxvirus infections has been established through studies involving strains of mice that are genetically resistant or genetically susceptible to EV infection. BALB/c mice succumbed to EV infection following inoculation with very small doses (<10 pfu) of virus, whilst C57BL/6 mice cleared and recover from high doses of virus (>10<sup>6</sup> pfu) (Karupiah, 1998). Following infection with EV, genetically resistant mice generated a Th1 cytokine profile (IFN- $\gamma$ , tumour necrosis factor- $\alpha$  [TNF- $\alpha$ ] and interleukin-2 [IL-2]) in the draining lymph node and spleen, whilst susceptible mice generated a Th2 cytokine profile, dominated by interleukin-4 (IL-4), with no IFN- $\gamma$ , TNF- $\alpha$  or IL-2 (Chaudhri *et al.*, 2004). The cytokine profile in resistant mice promoted the development of a potent cell-mediated adaptive immune response against the infection coupled with enhanced innate immune responses including increased NK cell activity. The development of a Th1-dominant response was responsible for the rapid control of virus replication in distal tissue and the subsequent survival of the mice.

The importance of cytokines in the control of poxvirus infections has also been investigated by systemically delivering or inhibiting cytokines during poxvirus infections, or using recombinant viruses expressing Th1 or Th2 cytokines. Expression of IL-4 (a key Th2 cytokine) in myxoma virus or EV enhanced the virulence of the recombinant virus and was able to overcome genetic resistance in rabbits and mice respectively, by suppressing CTL and innate immune responses (Jackson *et al.*, 2001; Kerr *et al.*, 2004). IL-4 expression in EV was also able to overcome pre-existing immunity to EV (Jackson *et al.*, 2001). VV expressing IL-4 also demonstrated increased virulence in mouse models, although infection was not lethal in immunocompetent mice (Andrew and Coupar, 1992; Sharma *et al.*, 1996). In contrast, VV recombinants expressing the Th1 cytokines IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-12 or IL-18 had reduced virulence and were cleared more rapidly than control viruses (Gherardi *et al.*, 2003; Hugin *et al.*, 1993; Karupiah *et al.*, 1990; Karupiah *et al.*, 1991; Kohonen-Corish *et al.*, 1990; Perera *et al.*, 2001; van Den Broek *et al.*, 2000). Similarly, systemic delivery of IFN- $\gamma$  or TNF- $\alpha$  enhanced innate and cell-mediated immune responses against EV (Atrasheuskaya *et al.*, 2004), whilst depletion of IFN- $\gamma$  resulted in a lethal infection in genetically resistant mice (Karupiah, 1998; Karupiah *et al.*, 1993).

#### 1.3.2.4 Summary

Current knowledge suggests that a Th1 cell-mediated immune response is critical for the rapid clearance of poxvirus infections in mice. Antibody plays an important role in the clearance of persistent poxvirus infections, but is not essential for survival from lethal challenge. The relative importance of CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses differs significantly between the closely related VV and EV infections. This suggests that each poxvirus disease may have different requirements for cell-mediated control of the infection. The importance of Th1 cell-mediated immunity in the control of poxviruses is further supported

by the fact that all Chordopoxviruses have evolved strategies to interfere with Th1 cytokines and cell-mediated immune responses including the secretion of anti-inflammatory proteins, down-regulation of host cell MHC-I expression, and cytokine and chemokine-binding proteins including those that that disrupt the function of IFN- $\alpha/\beta$ , IFN- $\gamma$ , TNF- $\alpha$ , IL-18 and IL-1 $\beta$  (Johnston and McFadden, 2003; Seet *et al.*, 2003; Smith *et al.*, 1998; Zuniga, 2002). Interference with Th1 cytokines and cell-mediated immunity is clearly advantageous to the *in vivo* replication of poxviruses.

### **1.3.3 Immunological memory and immune responses responsible for protection from poxvirus challenge**

A successful vaccine is capable of priming the adaptive immune system against specific components and molecules of a pathogenic organism to establish adequate levels of circulating antibody and antigen-specific memory cells that can prevent the induction of disease upon re-exposure to the pathogen. For a vaccine to be effective, it must induce adaptive immune responses that are able to quickly eliminate the invading pathogen before the induction of disease. These responses must be induced at the relevant site, be of the appropriate nature (antibodies, CTLs, Th1, Th2) and be of sufficient longevity to provide adequate protection from disease (Roitt, 1997; Zinkernagel, 2002).

The success of the smallpox eradication campaign is testament to the ability of poxvirus infections and vaccines to induce long-term protective memory responses. Following vaccination of humans with live VV vaccine, VV-specific antibody, CTL activity, lymphoproliferative activity and IFN- $\gamma$  production are detected within 10-14 days, with a predominant Th1 cytokine profile in VV-specific CD4<sup>+</sup> T cells (Amara *et al.*, 2004; Demkowicz and Ennis, 1993; Ennis *et al.*, 2002; Henderson and Moss, 1999). Immunity to

variola virus infection and smallpox is robust 50 years after vaccination with a single dose of live VV vaccine in infancy (Baxby, 2002; Cohen, 2001). Virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses are readily detectable 4 months after vaccination and for up to 70 years following vaccination (Demkowicz and Ennis, 1993; Demkowicz *et al.*, 1996; Hammarlund *et al.*, 2003). Similarly, following recovery from myxomatosis, rabbits are considered to have lifelong protection from further challenge, although they can become re-infected, albeit with drastically reduced symptoms usually limited to a lesion at the inoculation site (Fenner *et al.*, 1953; Kerr, 1997). However, not all poxvirus infections confer protection from re-infection. Most notably, immunity to orf virus is only transient in sheep and repeated re-infections are common, although with milder disease (Haig and Mercer, 1998).

Understanding of the specific immune mechanisms which are capable of conferring protection against challenge with a poxvirus is critical to the development of an effective poxvirus vaccine. Again, most data has been acquired from the VV/mouse and EV/mouse models that have permitted an elegant dissection of the immune mechanisms required for immunity. However, there is a great deal of conflicting data on the requirements for protection from challenge, and a definitive 'mechanism of immunity' has yet to be described.

#### *1.3.3.1 Antibody*

The importance of virus-specific antibody in protecting immunocompetent hosts from poxvirus infections has been demonstrated in many different poxvirus disease models. Humans with high levels of anti-VV antibodies were resistant to challenge with smallpox and low levels of VV neutralising antibody correlated with higher susceptibility to smallpox infection (Mack *et al.*, 1972; Sarkar *et al.*, 1975). Vaccinia immune globulin

(VIG) isolated from VV-immune individuals was of therapeutic benefit when administered to smallpox infected individuals and those suffering VV-induced disease after vaccination and is currently the only therapy available following Orthopoxvirus infection (CDC, 2001; Kempe, 1960; Kempe *et al.*, 1961).

Passive immunisation of mice with VV-immune serum confers complete protection from lethal VV infection, and a reduction in viral load (Boulter and Appleyard, 1973; Xu *et al.*, 2004). Passive immunisation with polyclonal or monoclonal antibodies directed against individual VV IMV or EEV antigens can also protect mice from lethal VV challenge (Galmiche *et al.*, 1999; Hooper *et al.*, 2000; Ramirez *et al.*, 2002). Transfer of EV-immune serum partially protects from EV challenge, though virus replication in the liver and spleen still occurs (Blanden, 1971) and passive immunisation of sheep with capripox-immune serum offers protection from capripox-induced disease (Kitching, 1986).

However, virus-specific antibody confers only limited or no protection in other poxvirus disease models. Passive transfer of orf-immune serum to sheep did not offer any significant protection from challenge (Buddle and Pulford, 1984; Mercer *et al.*, 1994). Similarly, passive immunisation of rabbits with immune serum protected only 15% of rabbits from lethal challenge with myxoma virus (Fenner and Marshall, 1954) and maternally-transferred antibody was unable to consistently protect young rabbits from infection or death from myxomatosis (Fenner and Marshall, 1954; Sobey and Conolly, 1975).

### 1.3.3.2 Cell-mediated immune mechanisms involved in immunity to poxvirus challenge

Although the importance of virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in recovery from poxvirus infections has been well established, the relative importance of virus-specific cell-mediated immune responses in preventing or ameliorating subsequent infections is not well understood and there are conflicting results in the literature.

Transfer of spleen cells from EV immune mice to naïve mice conferred significant protection from challenge, far in excess of that conferred by hyperimmune serum (Blanden, 1971; Blanden, 1974), indicating that virus specific cells alone, in the absence of anti-EV antibody, are capable of conferring protection from poxvirus challenge. However, depletion of CD4<sup>+</sup> or CD8<sup>+</sup> T cells in VV-immune mice did not alter disease pathogenesis upon challenge, and were only required for protection in the absence of VV-specific antibody (Belyakov *et al.*, 2003). In another study, VV-vaccinated CD4<sup>+</sup> T cell-deficient mice were not effectively protected from VV challenge (Wyatt *et al.*, 2004). In the absence of antibody, VV-immunised mice developed disease following lethal VV challenge (as measured by weight loss) but did not die, suggesting that memory T cell responses alone can protect from VV-induced death, but not disease, due to the time required for cell-mediated effector mechanisms to respond to infection (Belyakov *et al.*, 2003). Similarly, transfer of CD8<sup>+</sup> memory T cells from VV immune mice conferred high levels of protection from VV challenge, as seen by a 100-fold reduction in virus load (Xu *et al.*, 2004), indicating that CD8<sup>+</sup> memory responses are highly effective mediators of protection.

Outside of mouse models, the importance of cellular immune mechanisms in protection from poxviruses is uncertain due to the lack of inbred animal strains required for cellular transfer experiments. The only model studied in any detail is orf virus infection of sheep.

CD4<sup>+</sup> T cells are the predominant lymphocyte in orf lesions during primary and secondary infections (Jenkinson *et al.*, 1992; Jenkinson *et al.*, 1990), and depletion of CD4<sup>+</sup> cells in the early stages of re-infection resulted in increased lesion size and increased time to lesion resolution (Lloyd *et al.*, 2000). A similar, though smaller effect was noted upon depletion of CD8<sup>+</sup> T cells (Lloyd *et al.*, 2000).

The specific antigens against which cell-mediated responses are generated in animals following poxvirus infection have not been well studied. Responses against specific HLA-A2- or HLA-Db-restricted epitopes within five VV antigens have been measured in VV-infected mice and humans (VV Copenhagen ORFs H3L, B22R/C16L (common epitope), C7L, D6R and A26L) (Drexler *et al.*, 2003; Snyder *et al.*, 2004; Terajima *et al.*, 2003). Vaccination of mice with the H3L peptide did not confer protection from lethal VV challenge (Drexler *et al.*, 2003). However, vaccination of mice with the C7L or A26L peptides did confer CD8<sup>+</sup> T cell-dependant protection from VV-induced death, but not disease (Snyder *et al.*, 2004), indicating that memory CTL responses directed against a single VV epitope can confer protection in mice.

#### 1.3.2.3 Summary

These results suggest that virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells are not *absolutely* required for protection from lethal Orthopoxvirus challenge of mice if there is anti-Orthopoxvirus antibody present, but are essential in the absence of antibody. Cell-mediated immune responses alone are capable of preventing death but not virus-induced disease, presumably due to the time required for the proliferation and differentiation of memory T cells to effector CTLs (Esser *et al.*, 2003; Kaeck *et al.*, 2002). The relative importance of cell-mediated versus antibody-mediated protection in animals other than in the VV/mouse and

EV/mouse models is unknown. However, the absence of protection following passive transfer of antibody strongly suggests that cell-mediated immunity is critical in protecting sheep and rabbits from orf and myxoma viruses respectively. The importance of neutralising antibody in the VV/mouse model is most likely due to its ability to neutralise enough challenge virus, lowering the effective dose below lethal limits (Hooper *et al.*, 2000).

#### **1.4 POXVIRUS VACCINATION STRATEGIES**

Recovery from a poxvirus infection results in complete clearance of the pathogen and life-long immunity to disease caused by the virus (Esposito and Fenner, 2002). These characteristics make most poxvirus diseases readily preventable by vaccination, as clearance and recovery from the vaccine infection can confer the same life-long immunity.

Commercially available poxvirus vaccines are all live viruses. These take two forms – antigenically related poxviruses which are non-pathogenic but which confer cross-protection (heterologous vaccines), or pathogenic virus strains that have been attenuated by multiple passage in tissue culture resulting in random mutations and gene inactivation until a suitably safe but protective vaccine has been generated (homologous vaccines). Inactivated poxvirus vaccines are not effective in natural poxvirus disease models, even though high titres of neutralising antibody are generated (Fenner *et al.*, 1988).

Other experimental vaccination strategies that have proven effective in protecting mice from poxvirus challenge include subunit formulations consisting of individual poxvirus antigens or combinations of antigens delivered as protein or DNA vaccines, and non-replicating poxvirus strains. The characteristics, benefits and disadvantages of these



different vaccination strategies are described below. Vaccines against myxomatosis will be described in more detail in Section 1.5.

#### **1.4.1 Live attenuated poxvirus vaccines**

As live vaccine viruses can infect antigen presenting cells and produce antigens endogenously, they are proficient at inducing Th1-dominant cell mediated immune responses as well as neutralising antibodies against key viral antigens (Ahmed and Gray, 1996; Plotkin, 2003). For this reason, live attenuated poxvirus vaccines have proven successful in many poxvirus disease models because they are capable of inducing virus-specific cell-mediated immune responses that are critical for protection from poxvirus-induced disease (Section 1.3.3.2).

Some of the commercially available and experimental live, attenuated poxvirus vaccines are summarised in Table 1.3. Vaccines are available to prevent smallpox (Fenner *et al.*, 1988), fowlpox (Laidlaw and Skinner, 2004; Sarma and Sharma, 1988; Tripathy and Reed, 1997), orf (Buttner and Rziha, 2002; McInnes *et al.*, 2001; Nettleton *et al.*, 1996), lumpy skin disease (Capstick and Coakley, 1961; Davies, 1991; Kitching, 2003; Weiss, 1968), goatpox and sheeppox (Carn, 1993; Kitching, 2003) and myxomatosis (Section 1.5). The majority of these vaccines are homologous virus strains, except for VV-based smallpox vaccines (Fenner *et al.*, 1988) and the capripoxviruses, which are all antigenically related and cross protective (Carn, 1993).

Although most live poxvirus vaccines confer excellent protection, many have high complication rates. The best documented is VV vaccines in humans, as billions of doses were used during the smallpox eradication campaign (Fenner *et al.*, 1988). Following

Table 1.3 - Live attenuated poxvirus vaccines.

Pathogen and disease	Host	Vaccine	Nature of Vaccine	Efficacy	Problems	References
<b>ORTHOPOXVIRUSES</b>						
<b>Variola virus (smallpox)</b>	Humans	Dryvax, NYCBH, Lister, Ankara, Copenhagen and others	Live, replication-competent vaccinia virus	Excellent	Vaccine complications include fever, transmission to naive contacts, generalised vaccinia, progressive vaccinia (pf) <sup>*</sup> , and encephalitis (pf) <sup>*</sup> , and myopericarditis. Protection is dose-dependant. Live VV vaccines are potentially lethal to immunocompromised individuals.	CDC (2001) CDC (2004) Lane <i>et al.</i> (1969) Check (2004) Cassimatis <i>et al.</i> (2004) Maurer <i>et al.</i> (2003) Henderson and Moss (1999)
<b>Monkeypox virus (monkeypox)</b>	Cynomolgous monkeys ( <i>Macaca fascicularis</i> )	Dryvax	Live VV	Excellent	Lesion at the inoculation site persists for up to 3 weeks	Earl <i>et al.</i> (2004)
<b>Vaccinia virus</b>	Mice	Dryvax	Live VV	Excellent	Vaccine virus is lethal to immunocompromised mice	Wyatt <i>et al.</i> (2004) Belyakov <i>et al.</i> (2003)
<b>PARAPOXVIRUSES</b>						
<b>Orf virus (scabby mouth disease)</b>	Sheep and goats	Live orf virus	Tissue culture-attenuated virus strains	Poor	Vaccine can be transmitted to naive animals and cause disease. Vaccine strains have been source of disease outbreaks. Vaccine virus can infect humans. Poor long-term efficacy.	Haig and McInnes (2002) Buddle <i>et al.</i> (1984) Buddle and Pulford (1984) Gilray <i>et al.</i> (1998)
		D1701	Tissue culture-attenuated orf virus with deletions and duplications in the genome	Very good	Improved safety compared to other orf vaccine strains	Buttner and Rziha (2002) Nettleton <i>et al.</i> (1996) McInnes <i>et al.</i> (2001)

Table 1.3 continued

CAPRIOXVIRUS						
Sheeppox virus (sheeppox)	Sheep and goats	Live sheeppox virus or goatpox virus	Tissue culture-attenuated strains of sheeppox or goatpox	Very good	Vaccine-induced disease and transmission of vaccine virus to naive animals can occur.	Carr (1993)
Goatpox virus (goatpox)					Sheeppox and goatpox viruses confer cross-protection	Kitching (2003)
Lumpy skin disease virus (lumpy skin disease)	Cattle	Live lumpy skin disease virus (LSDV)	Tissue culture-attenuated lumpy skin disease virus	Very good	Efficacy is dependant on the breed of cattle. High incidence of vaccine-induced disease with some LSDV vaccine strains; not suitable for show-quality animals.	Hunter and Wallace (2001) Weiss (1968) Yeruham <i>et al.</i> (1994) Kitching (2003)
		Sheeppox virus	Live sheeppox virus	Moderate	Some sheeppox-derived vaccines are transmissible and pathogenic to sheep requiring herd separation. Outbreaks of LSDV reported in vaccinated herds.	Capstick and Coakley (1961) Davies (1991) Hunter and Wallace (2001)
FOWLPOXVIRUSES						
Fowlpox	Chickens	Fowlpox	Various tissue culture-attenuated fowlpox virus strains	Excellent Poor against 'variant fowlpox'	Some vaccine strains contain reticuloendothelium virus within genome.	Tripathy and Reed (1997) Sarma and Sharma (1988) Laidlaw and Skinner (2004) Singh <i>et al</i> (2000)

\* - pf, potentially fatal complication

vaccination, most recipients developed very mild complications including fever and swollen and tender lymph nodes (Henderson and Moss, 1999). More serious, potentially lethal complications included generalised vaccinia, progressive vaccinia, postvaccinal encephalitis, myopericarditis and transmission of the vaccine virus to naïve contacts (CDC, 2001; Henderson and Moss, 1999; Lane *et al.*, 1969; Maurer *et al.*, 2003). Fatalities, primarily due to progressive vaccinia and nervous system complications were responsible for approximately 1 death per million vaccinees during the 1960's (CDC, 2001). The recent vaccination campaign in the US highlighted that adverse effects of VV vaccination, especially myopericarditis, are still common (Cassimatis *et al.*, 2004; CDC, 2004; Check, 2004). Today, the use of traditional live VV vaccines poses a significant risk to immunosuppressed individuals (Bartlett *et al.*, 2003; Bartlett, 2003a; Bartlett, 2003b; Redfield *et al.*, 1987), and immunosuppression due to HIV infection is much more widespread than when smallpox vaccination was discontinued in the early 1970's.

Other live poxvirus vaccines have similar problems. Orf virus vaccine strains have been responsible for outbreaks of scabby mouth disease (Gilray *et al.*, 1998). Capripoxvirus vaccines can induce disease and severe reactions at the inoculation site, and many are not considered suitable for animals of show quality (Carn, 1993; Yeruham *et al.*, 1994). Transmission of the vaccines have been reported, especially where sheeppox virus has been utilised as a heterologous LSDV vaccine, as this vaccine virus is still pathogenic for naive sheep (Capstick and Coakley, 1961; Davies, 1991).

#### **1.4.2 Subunit poxvirus vaccines**

Vaccines consisting of whole inactivated viruses or components of viruses are highly effective in many different disease models. Subunit vaccines are considered much safer

than live vaccines due to their non-replicating nature (Plotkin, 2003). The parenteral delivery of these vaccines tend to generate Th2 and potent neutralising antibody responses, and are highly effective against viruses where neutralising antibody is protective. However, non-live vaccines, especially protein vaccines, generate no or poor CTL responses, and are not effective for viruses where CTL responses are critical for protection (Zinkernagel, 2002).

Protein subunit poxvirus vaccines, consisting of individual poxvirus antigens delivered as a protein vaccine have had some success in experimental animal models (Table 1.4). The 14 kDa IMV fusion protein A27L confers complete protection from lethal VV challenge in mice when delivered as a protein vaccine (Demkowicz *et al.*, 1992; Lai *et al.*, 1991b). The VV core antigens A4L and A10L were also able to confer partial protection (Demkowicz *et al.*, 1992). Goats have been partially protected from challenge with virulent goatpox virus following vaccination with the P32 protein (homologous to VV H3L), although all challenged animals still developed symptoms of disease (Carn *et al.*, 1994).

DNA vaccines have proven an effective means of identifying the protective antigens of Orthopoxviruses (Table 1.4). In its most basic form, a DNA vaccine consists of a bacterial plasmid expressing a protein antigen under the control of a constitutive mammalian promoter (Lai and Bennet, 1998). When delivered to the host, the plasmid is taken up by host cells, the antigen gene transcribed and translated and the resultant foreign protein is processed via cellular proteosomic pathways and presented to immune cells in the context of MHC-I (Gurunathan *et al.*, 2000; Weiner and Kennedy, 1999). As the antigenic protein is synthesised and processed within host cells, DNA vaccines are one of the few non-live vaccination methods capable of inducing potent Th1/cell-mediated immune responses as

**Table 1.4 - Subunit poxvirus vaccines.** The protective efficacy of subunit vaccines have been evaluated in mice challenged with VV, monkeys challenged with monkeypox and goats challenged with goatpox.

Antigen and Delivery System	Efficacy (% survival) <sup>a</sup>	Comments	References
<b>Lethal vaccinia virus challenge of BALB/c mice</b>			
A4L protein	50		Demkowicz <i>et al.</i> (1992)
A10L protein	75		Demkowicz <i>et al.</i> (1992)
A27L protein	100		Lai <i>et al.</i> (1991b) Demkowicz <i>et al.</i> (1992)
A27L DNA vaccine	80, 10		Pulford <i>et al.</i> (2004) Hooper <i>et al.</i> (2003)
B5R DNA vaccine	85, 100		Galmiche <i>et al.</i> (1999) Pulford <i>et al.</i> (2004)
A33R DNA vaccine	100, 67, 70		Galmiche <i>et al.</i> (1999) Pulford <i>et al.</i> (2004) Hooper <i>et al.</i> (2000)
A36R DNA vaccine	50, 60		Galmiche <i>et al.</i> (1999) Pulford <i>et al.</i> (2004)
A34R DNA vaccine	15, 0		Galmiche <i>et al.</i> (1999) Pulford <i>et al.</i> (2004)
A13L DNA vaccine	0		Pulford <i>et al.</i> (2004)
D8L DNA vaccine	50		Pulford <i>et al.</i> (2004)
H3L DNA vaccine	33		Pulford <i>et al.</i> (2004)
L1R DNA vaccine	0, 80		Pulford <i>et al.</i> (2004) Hooper <i>et al.</i> (2000)
L1R + A33R DNA vaccine	90	Weight loss profile and survival rate identical to Dryvax-vaccinated mice	Hooper <i>et al.</i> (2000)
A27L + B5R DNA vaccine	70		Hooper <i>et al.</i> (2003)
B5R + L1R DNA vaccine	100		Hooper <i>et al.</i> (2003)
B5R + A33R DNA vaccine	80		Hooper <i>et al.</i> (2003)
A33R + B5R + A27L + L1R DNA vaccine	100	Less weight loss than Dryvax-vaccinated mice	Hooper <i>et al.</i> (2003)
CD8 <sup>+</sup> HLA-A2-restricted epitope from VV C7L	80		Snyder <i>et al.</i> (2004)
<b>Monkeypox virus challenge of rhesus macaques</b>			
VV A33R, + B5R + A27L + L1R DNA vaccine	100	Some secondary signs of disease	Hooper <i>et al.</i> (2004)
VV L1R DNA vaccine	100	More severe disease than seen in monkeys which received four-antigen combination	Hooper <i>et al.</i> (2004)
<b>Goatpox virus challenge of British white cross goats</b>			
P32 (VV H3L) protein	100	All animals developed disease, but survived	Carn <i>et al.</i> (1994)

a - Each number represents the result of a separate experiment

well as humoral immune responses (Gurunathan *et al.*, 2000; Shedlock and Weiner, 2000; Takashima and Morita, 1999; Weiner and Kennedy, 1999).

DNA vaccines expressing EEV or IMV antigens have proven highly effective at protecting mice from VV challenge, although with varying results between antigens and experiments (Table 1.4; Galmiche *et al.*, 1999; Hooper *et al.*, 2000; Hooper *et al.*, 2003; Pulford *et al.*, 2004). The four-gene combination of the VV genes L1R, A33R, B5R and A27L has proven effective at protecting monkeys from lethal monkeypox challenge (Hooper *et al.*, 2004), and in mice, this combination proved more effective than vaccination with live VV (Hooper *et al.*, 2003).

It is important to note that the majority of research on poxvirus subunit vaccines has been conducted in the VV/mouse model, which as discussed previously, is not a natural poxvirus disease model. Mice are very well protected from lethal VV challenge by VV-neutralising antibodies (Boulter and Appleyard, 1973; Xu *et al.*, 2004), which is not the case with most other poxvirus diseases (Buddle and Pulford, 1984; Cohen, 2001; Fenner and Marshall, 1954; Mercer *et al.*, 1994). High titres of neutralising antibody directed against these IMV and EEV antigens would be capable of neutralising enough challenge virus to lower the effective dose below lethal limits (Hooper *et al.*, 2000). However, many of these DNA vaccines had protective efficacy despite no VV neutralising antibody activity after vaccination (Galmiche *et al.*, 1999; Pulford *et al.*, 2004). This suggests antigen-specific cell-mediated mechanisms are conferring protection for at least some of these antigens, although the magnitude and phenotype of cell-mediated responses induced by DNA vaccines have not been reported. It is unknown whether DNA vaccines expressing IMV,

EEV, structural or enzymatic antigens will be protective in natural poxvirus disease models where virus-specific cell-mediated immunity is critical for protection.

### 1.4.3 Replication-deficient poxvirus vaccines

Replication-deficient smallpox vaccines have been investigated due to the increased safety of these vaccines in immunocompromised individuals. These viruses are unable to replicate in host cells due to the deletion of key viral host range genes, with viral replication terminating before the assembly of new virion particles (Blanchard *et al.*, 1998). As these viruses are incapable of productive infection *in vivo*, they cannot disseminate beyond the inoculation site and induce disease. However, their ability to express a large number of viral antigens *in vivo* before termination of the replication cycle results in the effective delivery of a wide variety of poxvirus antigens and epitopes (McCurdy *et al.*, 2004).

Only two replication-deficient poxvirus vaccines have been evaluated as protective vaccines, the VV strains modified vaccinia virus Ankara (MVA) and NYVAC (Table 1.5). MVA was attained after 570 passages of the VV strain Ankara in chicken embryo fibroblasts (Antoine *et al.*, 1998). This resulted in the splitting or deletion of 25 open reading frames including the important immunomodulatory genes C23L (chemokine binding protein), C19L (TNF- $\alpha$  receptor homologue), Serp-1, B4R (homologous to myxoma virus anti-apoptotic factor M-T5) and host-range genes including K1L (Antoine *et al.*, 1998). Expression of the interferon- $\gamma$  binding protein and interferon- $\alpha/\beta$  binding proteins was not detected (Blanchard *et al.*, 1998). NYVAC was constructed by the targeted deletion of 18 host range, immunomodulatory and enzymatic genes (Tartaglia *et al.*, 1992). The effect of these mutations and deletions in both viruses is a highly restricted host cell range. MVA and NYVAC cannot productively infect most mammalian cell lines



Table 1.5 - Replication-deficient *Orthopoxvirus* vaccines.

Pathogen and disease	Host	Vaccine	Nature of Vaccine	Efficacy	Safety	References
<b>Variola virus (smallpox)</b>	Humans	MVA	Replication-deficient VV strain, produced by 570 passages of VV Ankara in chicken embryo fibroblasts	Unknown	Safe in immunocompromised individuals. Efficacy against smallpox challenge has never been assessed.	Rosenthal <i>et al.</i> (2001) Smith and McFadden (2002) Mayr <i>et al.</i> (1978)
<b>Monkeypox virus</b>	Cynomolgous monkeys ( <i>Macaca fascicularis</i> )	Two doses of MVA ( $10^8$ pfu)		Very good	Not as effective as a single dose of Dryvax	Earl <i>et al.</i> (2004)
		MVA ( $10^8$ pfu) + Dryvax boost		Excellent	Smaller Dryvax-induced lesion	Earl <i>et al.</i> (2004)
<b>Vaccinia virus</b>	Mice	MVA		Very good	Protection was dose-dependant, and not as effective as Dryvax when challenged with $10^7$ pfu of VV. MVA is safe in immunocompromised mice.	Wyatt <i>et al.</i> (2004) Belyakov <i>et al.</i> (2003)
<b>Cowpox virus</b>	Rabbits	MVA		Poor after a single dose Excellent after a boost	Required a boost to provide good protection. Intramuscular injection was more effective than subcutaneous infection.	Munz <i>et al.</i> (1993)
<b>Vaccinia virus</b>	Mice and rabbits	NYVAC	Replication-deficient VV strain produced by the targeted deletion of 18 genes	Very good	Protection was dose-dependant. Safe in immunocompromised mice and rabbits. NYVAC is cleared more quickly than replication-competent VV from rabbit skin. NYVAC did not induce ulceration of rabbit skin.	Tartaglia <i>et al.</i> (1992) Belyakov <i>et al.</i> (2003)

*in vitro*, but can grow to high titres in some avian cell lines such as chick embryo fibroblasts (Blanchard *et al.*, 1998).

MVA is effective in preventing lethal VV in mice (Belyakov *et al.*, 2003; Wyatt *et al.*, 2004), cowpox infection in rabbits (Munz *et al.*, 1993) and monkeypox in cynomolgous monkeys (Earl *et al.*, 2004). The range of anti-VV immune responses induced by two doses of MVA in mice and monkeys was comparable to that induced by standard replication-competent VV vaccines (Belyakov *et al.*, 2003; Earl *et al.*, 2004; Wyatt *et al.*, 2004). Two doses of MVA were capable of protecting monkeys from lethal monkeypox, although most animals developed very mild disease (Earl *et al.*, 2004). A single dose of MVA followed by DryVax significantly reduced the size of the lesion induced by DryVax, and offered complete protection from disease, comparable to a single DryVax inoculation (Earl *et al.*, 2004). However, their efficacy in protecting humans from smallpox has not been evaluated (Rosenthal *et al.*, 2001; Smith and McFadden, 2002).

MVA and NYVAC have proven safe and effective at inducing protective immunity against poxvirus challenge in immunocompromised mice, rabbits and monkeys, even at very high doses (Edghill Smith *et al.*, 2003; Wyatt *et al.*, 2004). MVA was used extensively in Germany during the smallpox eradication campaign with no reported side effects, even amongst susceptible populations (Mayr *et al.*, 1978). Taken together, these data suggest that replication-deficient vaccines would be safe to use in immunocompromised humans.

## **1.5 MYXOMATOSIS VACCINES**

Currently available myxomatosis vaccines are all attenuated live viruses. In Europe, there are several widely used myxomatosis vaccines of two forms – heterologous vaccines using

the antigenically related poxvirus, rabbit fibroma virus (RFV), or homologous vaccines using tissue culture-attenuated strains of myxoma virus (Marlier *et al.*, 2000; Vautherot *et al.*, 1997). The details of myxomatosis vaccines and their protective efficacy are summarised in Table 1.6.

RFV-based vaccines are widely used in the United Kingdom. Protection from myxomatosis is generally limited and short-lived, and dependent on the RFV strain used. Vaccination with RFV strain OA induced only a small lesion on rabbits and the protection conferred was extremely limited and short-lived. When challenged with virulent myxoma virus 3 or 5 months after vaccinations, seven of nine of rabbits developed severe myxomatosis and two died (Fenner and Woodroffe, 1954). The Boerlage strain of RFV induced a more severe primary lesion following vaccination (up to 40 mm in diameter) although no vaccine-induced disease was noted in rabbits as young as 14 days. Following challenge with high doses of virulent myxoma virus, a significant level of protection was observed up to 12 months following vaccination (Fenner and Woodroffe, 1954). However, commercially available RFV vaccines appear to offer only poor protection against myxoma virus challenge (Marlier *et al.*, 2000).

Several homologous myxomatosis vaccines have been developed which are widely used in Continental Europe (Gorski *et al.*, 1994; Vautherot *et al.*, 1997). These vaccines are field strains of virus that were passaged *in vitro* until a suitably attenuated strain of virus was obtained (Brun *et al.*, 1981; Jacotot *et al.*, 1967; McKercher and Saito, 1964; Saurat *et al.*, 1978). The widely used French vaccine SG33 was very effective at preventing lethal myxomatosis, but protection was relatively short lived. By 12 months after vaccination, 50% of rabbits succumbed to lethal infection (Picavet *et al.*, 1989; Saurat *et al.*, 1978). At

Table 1.6 - Myxomatosis vaccines.

Vaccine virus and strain	Vaccine characteristics	Efficacy	Safety	References
<b>Heterologous myxomatosis vaccines</b>				
RFV OA	Antigenically related, non-pathogenic Leporipoxvirus	Poor	Small vaccine-induced lesion (<20 mm). Very safe in young rabbits. Poorly protective by 3 months after vaccination.	Fenner and Woodroffe (1954)
RFV Boerlage	Antigenically related, non-pathogenic Leporipoxvirus	Good	Large vaccine-induced lesion (40 mm). Safe in young rabbits. Better long-term protection than OA.	Fenner and Woodroffe (1954)
Nobivac Myxo (Intervet RFV vaccine)	Antigenically related, non-pathogenic Leporipoxvirus	Very poor	Small vaccine-induced lesion. Very safe in young rabbits. Very poor protection upon challenge (only 30% survival).	Marlier <i>et al.</i> (2000)
<b>Homologous myxomatosis vaccines</b>				
Myxoma virus SG33	Tissue culture-attenuated Brazilian myxoma virus strain	Good	Vaccine-induced disease and generalised immunosuppression in some young rabbits (pf)*; poor long-term efficacy.	Saurat <i>et al.</i> (1978) Vautherot <i>et al.</i> (1997) Brun <i>et al.</i> (1981)
Myxoma virus Myxovac M	Tissue culture-attenuated Brazilian myxoma virus strain	Excellent	Vaccine virus disseminated to distal tissues including eyelids, testis and secondary skin sites.	Gorski <i>et al.</i> (1994)
Attenuated myxoma virus	Tissue culture-attenuated Californian myxoma virus	Good	Fever during vaccine infection. Vaccine transmissible to naive animals.	McKercher and Saito (1964)
<b>Prime-boost with heterologous and homologous vaccines</b>				
RFV + SG33 boost	RFV followed by an SG33 boost 4 weeks	Good	Better protection than RFV alone, but 20% of rabbits developed serious disease upon challenge.	Vautherot <i>et al.</i> (1997) Marlier <i>et al.</i> (2000)

\* pf - potentially fatal complication

higher doses (>2000 pfu), SG33 is capable of inducing mild secondary signs of myxomatosis in 8-12 week old rabbits. Similarly, other myxoma virus vaccines can cause side effects such as fever (McKercher and Saito, 1964), and can disseminate to distal tissues (Gorski *et al.*, 1994).

Current attenuated myxoma virus vaccine strains can induce mild myxomatosis and an immunosuppressed state, especially in younger animals, which can result in the establishment of lethal secondary bacterial infections (Brun *et al.*, 1981; Vautherot *et al.*, 1997), and reversion to virulence has been reported (Jacotot *et al.*, 1967). A ‘recommended’ myxomatosis vaccination regime to reduce the incidence of vaccine-induced disease involves vaccination with the heterologous RFV vaccine first, followed by a boost with a homologous myxoma virus vaccine (Marlier *et al.*, 2000; Vautherot *et al.*, 1997). However, this vaccination regime only offers moderate protection from disease (Marlier *et al.*, 2000).

Another field of myxomatosis vaccine research is transmissible myxomatosis vaccines for use in wildlife conservation. Researchers in Spain have been developing a transmissible myxoma virus/rabbit haemorrhagic disease virus (RHDV) vaccine to protect wild rabbit populations from both viruses (Bertagnoli *et al.*, 1996). In some parts of Europe, rabbits form the primary food source for several highly endangered predators including the lynx and imperial eagle. Since the outbreak of RHDV, rabbit populations have dropped to very low levels, adversely affecting the populations of the predators (Ferrer and Negro, 2004; Rodriguez and Delibes, 2002). A myxoma virus strain isolated from the wild in Spain has been modified to encode the RHDV capsid protein VP60, and confers complete protection from both myxoma virus and RHDV challenge (Bertagnoli *et al.*, 1996). This recombinant

vaccine has proven to be very safe in wild rabbits under laboratory conditions (Torres *et al.*, 2000), and has at least limited horizontal transmissibility of protection in laboratory and field trials (Barcena *et al.*, 2000; Torres *et al.*, 2001).

## 1.6 SUMMARY AND AIMS OF THIS PROJECT

Despite the need for more effective poxvirus vaccines to combat diseases such as goatpox, sheepox, scabby mouth disease, lumpy skin disease, myxomatosis and the potential re-emergence of smallpox, the currently available poxvirus vaccines are all live, attenuated vaccines that have an unacceptably high incidence of side-effects or poor efficacy. This project has evaluated the efficacy of novel poxvirus vaccine designs in protecting rabbits from challenge with myxoma virus - a natural host/pathogen model. This model allows us to determine the characteristics of a vaccine that is capable of protecting a host from a highly lethal, naturally transmissible poxvirus, whilst causing minimal side effects.

The vaccine candidates were evaluated using the following criteria for an ‘ideal’ myxoma virus vaccine or vaccination strategy:

1. The vaccine itself would cause no illness or distress in vaccinated animals, especially younger (8-12 week old) animals, including any long-term scarring caused by vaccine-induced clinical manifestations (vaccine **acceptability**).
2. The vaccine would confer complete protection (total absence of any clinical manifestations of disease) from challenge with virulent strains of myxoma virus (vaccine **efficacy**).
3. The vaccine would be unable to transmit between a recently vaccinated animal and other animals, either by direct contact or via arthropod vectors (**non-transmissibility**).

In Chapter 3, the protective efficacy of DNA vaccines expressing single and multiple myxoma virus antigens and rabbit cytokines is evaluated. Chapter 4 describes the construction of three live, attenuated myxoma virus vaccines by the deletion of key viral immunomodulatory genes. These vaccines were evaluated for their safety and efficacy. This poses the question: can a live, attenuated vaccine be constructed such that it is completely safe, but still be capable of conferring complete protection from challenge? One of these vaccine strains proved to be highly efficacious and safe in rabbits, and it was studied in greater detail. In Chapter 5, the pathogenesis of this vaccine was studied to determine the following: the potential transmissibility of this vaccine between animals; the degree of dissemination of the vaccine virus that occurred *in vivo*; and the degree of tissue damage that occurred at the site of inoculation and the draining lymph node. In Chapter 6, the safety and efficacy of this live, attenuated virus was evaluated in 8 and 12 week old rabbits. Finally, a replication-deficient myxoma virus vaccine was constructed by the deletion of a key host-range gene, and the virus evaluated for its protective efficacy and safety, both alone and as part of a prime-boost regimen (Chapter 7).

## **Chapter 2**

# **Materials and Methods**



## CHAPTER 2 - MATERIALS AND METHODS

### 2.1 MOLECULAR BIOLOGY METHODS

#### 2.1.1 Growth and storage of bacterial cultures

Unless otherwise indicated, bacterial cultures were grown overnight at 37°C. Luria-Bertani (LB) broth (1% tryptone [Bacto, Australia], 0.5% yeast extract [Bacto, Australia] and 1% NaCl [BDH, Australia]) was used for liquid cultures and LB agar (LB broth with 1.5% agar [Bacto, Australia]) was used for the plating of bacteria. Liquid cultures were incubated in a shaking incubator at approximately 200 rpm. Media was supplemented with 100 µg/mL of ampicillin (Sigma, Australia) or 30 µg/mL of kanamycin (Sigma, Australia) if required. Cultures were stored at 4°C for the short term. For long term storage, bacterial cultures were grown overnight in LB broth, mixed with an equal volume of glycerol (BDH, Australia) and stored at -80°C.

#### 2.1.2 Cloning into plasmids

##### 2.1.2.1 Restriction endonuclease digestion of DNA

DNA was digested with appropriate restriction endonucleases for analytical purposes and to generate cohesive ends for cloning. For cloning purposes, a reaction was prepared containing 10-20 units of the appropriate restriction endonuclease, 5 µL of the corresponding 10x incubation buffer, 20 µL plasmid miniprep DNA or PCR product DNA (concentration not determined) and MilliQ water to a final volume of 50 µL. The mixture was incubated at 37°C for 2-4 h. The required fragments were isolated by agarose gel electrophoresis (Section 2.1.5), and purified (Section 2.1.6).

### 2.1.2.2 Dephosphorylation of linearised vector

To prevent self-ligation, linearised plasmids used during cloning were dephosphorylated. Following digestion with the appropriate restriction endonuclease, 2 units of shrimp alkaline phosphatase (Roche, Germany) was added to the restriction reaction and the mixture incubated for a further 30 min at 37°C. Dephosphorylated, linear vector was isolated by agarose gel electrophoresis (Section 2.1.5) and purified (Section 2.1.6).

### 2.1.2.3 Ligations

Ligation reactions contained 1 µL of 10x T4 DNA ligase buffer (MBI, USA), 1 unit of T4 DNA ligase (MBI, USA), 1 µg of dephosphorylated vector DNA, insert DNA and MilliQ water to a final volume of 10 µL. An approximate insert DNA : vector DNA molar ratio of 5 was used for all ligation reactions. The reaction was incubated at 16°C overnight. If not used immediately for transformation, ligation reactions were stored at -20°C.

### 2.1.2.4 TA cloning

The pGEM-T Easy Cloning Kit (Promega, USA) was used for the direct cloning of *Taq* DNA polymerase-amplified PCR products. This enzyme adds a single 3'-A residue to the PCR product regardless of the template's sequence. The linear vector possesses 3' single T overhangs that allow for the efficient cloning of these PCR products.

Ligation reactions were set up according to the manufacturer's instructions with modifications. To 7 µL of agarose gel electrophoresis-purified PCR product (Section 2.1.6) was added 1 µL of 10x T4 ligase buffer, 1 µL T4 ligase and 1 µL of pGEM-T Easy vector. This mixture was incubated at 16°C overnight, and transformed into *E. coli* as described in Section 2.1.2.6. If not used immediately, the ligation reaction was stored at -20°C.

### 2.1.2.5 Preparation of electrocompetent cells

To prepare electrocompetent cells, a glycerol stock of the required *E. coli* strain was streaked onto LB agar and grown overnight at 37°C. A single colony was selected and grown overnight in LB broth at 37°C. The next morning, this culture was diluted 1:20 in 200 mL of fresh LB broth in a 500 mL conical flask and incubated with shaking at 37°C until mid-log phase (3 h). The bacteria were pelleted by centrifugation at 5000 rpm for 10 min at 4°C (Sorvall RC5+ centrifuge, GSA rotor). The supernatant was discarded and the bacteria gently resuspended in 200 mL of ice-cold (1°C) sterile MilliQ water. The bacteria were pelleted at 5000 rpm for 10 min, and the supernatant discarded. The pellet was again resuspended in 100 mL of cold sterile MilliQ water and pelleted by centrifugation. The supernatant was discarded, and the bacteria suspended in 4 mL of cold sterile 10% glycerol. The bacteria were pelleted once more, the supernatant discarded and the bacteria suspended in 400 µL of cold sterile 10% glycerol. The bacterial suspension was divided up into 40 µL aliquots on ice in microfuge tubes, and stored at -80°C.

### 2.1.2.6 Transformation of electrocompetent cells

Electrocompetent *E. coli* was transformed by electroporation using a BioRad GenePulser II. The competent bacterial cells were thawed on ice. The DNA to be transformed into the cells (1-2 µL of plasmid miniprep or ligation reaction) was mixed with the cells, and the entire contents transferred to a BioRad GenePulser 0.2 cm electroporation cuvette that had been chilled on ice. The suspension was subjected to electroporation at 2.5 kV, 200 ohms and 25 µF. Immediately following this, 1 mL of chilled LB broth was added, and the suspension transferred back into the original tube. The cell suspension was incubated at 37°C for 1 h. After incubation, 50 µL of the cells were plated onto LB agar plates

supplemented with 100 µg/mL of ampicillin or 30 µg/mL of kanamycin. IPTG and X-gal were added to the agar plates if  $\alpha$ -complementation was being carried out (Section 2.1.2.7).

#### *2.1.2.7 $\alpha$ -complementation*

The vectors used in this project often allowed the use of  $\alpha$ -complementation to isolate those clones that contained insert DNA.  $\alpha$ -complementation selection plates were prepared by spreading 20 µL each of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG; 20 mg/mL [Sigma, Australia]) and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal; 20 µg/mL [Sigma, Australia]) onto LB agar plates containing ampicillin (100 µg/mL). The transformed bacteria (50 µL) were spread on the surface of these plates and incubated for 16-20 h at 37°C. White colonies were selected for further analysis by transfer to a fresh LB-ampicillin agar plate or broth. These cultures underwent miniprep (Section 2.1.3.1) and restriction analysis (Section 2.1.2.1).

### **2.1.3 Plasmid DNA preparation**

The plasmids used in this study are listed in Table 2.1.

#### *2.1.3.1 Small scale plasmid DNA preparation (Miniprep)*

Plasmid DNA was prepared using the Qiagen Spin Miniprep Kit according to the manufacturer's instructions. Briefly, the bacterial strain containing the plasmid of interest was streaked onto an LB agar plate supplemented with the appropriate antibiotic and incubated overnight at 37°C. A single colony was selected, placed into 5 mL of LB broth supplemented with the appropriate antibiotic and incubated overnight at 37°C. The next morning, 1.5 mL of this culture was poured into a microfuge tube and the bacteria pelleted in a microcentrifuge at 13,000 rpm for 60 sec (Eppendorf 5415C centrifuge). The

**Table 2.1** – Cloning vectors and plasmids used in this study.

Plasmid / Vector	Comments	Source / Reference
<b>Vectors</b>		
<b>pDual2+</b>	DNA vaccine vector containing two expression cassettes controlled by CMV-IE promoters and BGH-pA terminators, derived from pcDNA3.1+; Amp <sup>R</sup>	Scott Thompson, JCSMR Figure 3.1
<b>pGEM-T Easy</b>	TA-cloning vector for the cloning of <i>Taq</i> DNA polymerase amplified PCR products; Amp <sup>R</sup> , <i>LacZ</i> <sup>+</sup>	Promega
<b>pET30a<sup>+</sup></b>	Cloning vector for the production of recombinant protein in $\lambda$ phage lysogenic <i>E. coli</i> , under the T7- <i>lac</i> promoter system; Kan <sup>R</sup>	Novagen
<b>pKS<sup>+</sup>Bluescript</b>	Cloning vector containing extensive multiple cloning site; Amp <sup>R</sup> , <i>LacZ</i> <sup>+</sup>	
<b>DNA Vaccine Plasmids</b>		
<b>pDual-HA</b>	pDual2+ containing the haemagglutinin gene from influenza A/Puerto Rico/8 in the first expression cassette	This study (Chapter 3)
<b>pDual-IL2</b>	pDual2+ containing the rabbit interleukin-2 cDNA cloned into the second expression cassette	This study (Chapter 3)
<b>pDual-IL4</b>	pDual2+ containing the rabbit interleukin-4 cDNA cloned into the second expression cassette	This study (Chapter 3)
<b>pDual-55R</b>	pDual2+ containing the myxoma virus antigen gene, M055R in the first expression cassette	This study (Chapter 3)
<b>pDual-55R-IL2</b>	pDual-55R containing the rabbit interleukin-2 cDNA cloned into the second expression cassette.	This study (Chapter 3)
<b>pDual-55R-IL4</b>	pDual-55R containing the rabbit interleukin-4 cDNA cloned into the second expression cassette	This study (Chapter 3)
<b>pDual-73R</b>	pDual2+ containing the myxoma virus antigen gene, M073R in the first expression cassette	This study (Chapter 3)

Table 2.1 continued

<b>pDual-73R-IL2</b>	pDual-73R containing the rabbit interleukin-2 cDNA cloned into the second expression cassette	This study (Chapter 3)
<b>pDual-73R-IL4</b>	pDual-73R containing the rabbit interleukin-4 cDNA cloned into the second expression cassette	This study (Chapter 3)
<b>pDual-107L</b>	pDual2+ containing the myxoma virus antigen gene, M107L in the first expression cassette	This study (Chapter 3)
<b>pDual-107L-IL2</b>	pDual-107L containing the rabbit interleukin-2 cDNA cloned into the second expression cassette	This study (Chapter 3)
<b>pDual-107L-IL4</b>	pDual-107L containing the rabbit interleukin-4 cDNA cloned into the second expression cassette	This study (Chapter 3)
<b>pDual-115L</b>	pDual2+ containing the myxoma virus antigen gene, M115L in the first expression cassette	This study (Chapter 3)
<b>pDual-115L-IL2</b>	pDual-115L containing the rabbit interleukin-2 cDNA cloned into the second expression cassette	This study (Chapter 3)
<b>pDual-115L-IL4</b>	pDual-115L containing the rabbit interleukin-4 cDNA cloned into the second expression cassette.	This study (Chapter 3)
<b>pDual-121R</b>	pDual2+ containing the myxoma virus antigen gene, M121R in the first expression cassette	This study (Chapter 3)
<b>pDual-121R-IL2</b>	pDual-121R containing the rabbit interleukin-2 cDNA cloned into the second expression cassette	This study (Chapter 3)
<b>pDual-121R-IL4</b>	pDual-121R containing the rabbit interleukin-4 cDNA cloned into the second expression cassette	This study (Chapter 3)
<b>Recombinant Protein Synthesis Plasmids</b>		
<b>pET-55R</b>	pET30a+ containing the myxoma virus antigen gene M055R, cloned in frame with the 5' purification tags, between the <i>EcoRI</i> and <i>XhoI</i> sites	This study (Chapter 3)
<b>pET-73R</b>	pET30a+ containing the myxoma virus antigen gene M073R, cloned in frame with the 5' purification tags, between the <i>EcoRI</i> and <i>XhoI</i> sites	This study (Chapter 3)

Table 2.1 continued

<b>pET-115L</b>	pET30a+ containing the myxoma virus antigen gene M115L, cloned in frame with the 5' purification tags, between the <i>EcoRI</i> and <i>XhoI</i> sites	This study (Chapter 3)
<b>pET-121R</b>	pET30a+ containing the myxoma virus antigen gene M121R, cloned in frame with the 5' purification tags, between the <i>EcoRI</i> and <i>XhoI</i> sites	This study (Chapter 3)
<b>Plasmids used in the construction of recombinant myxoma viruses</b>		
<b>pKS<sup>+</sup>ΔM10L/M11Lgpt</b>	pKS <sup>+</sup> Bluescript containing two regions of the M10L/M11L locus of the myxoma virus strain, Uriarra between which is cloned the <i>gpt</i> gene of <i>E. coli</i> under the control of the poxvirus promoter p7.5	This study (Chapter 4) Figure 4.1
<b>pKS<sup>+</sup>ΔM-T7gpt</b>	pKS <sup>+</sup> Bluescript containing two regions of the MT-7 locus of the myxoma virus strain, Uriarra between which is cloned the <i>gpt</i> gene of <i>E. coli</i> under the control of the poxvirus promoter p7.5	This study (Chapter 4) Figure 4.3
<b>pKS<sup>+</sup>ΔM-T7gus</b>	pKS <sup>+</sup> Bluescript containing two regions of the MT-7 locus of the myxoma virus strain, Uriarra (identical to pKS <sup>+</sup> ΔMT-7gpt) between which is cloned the <i>E. coli</i> gene <i>gus</i> under control of the poxvirus promoter pE/L	This study (Chapter 4) Figure 4.3
<b>pKS<sup>+</sup>ΔM063Rgptgus</b>	pKS <sup>+</sup> Bluescript containing two regions of the M063R locus of the myxoma virus strain, Uriarra between which is cloned the fusion gene <i>gpt-gus</i> , both derived from <i>E. coli</i> , and under the control of the poxvirus promoter pE/L	This study (Chapter 7) Figure 7.1

supernatant was discarded, another 1.5 mL of culture added to the tube, the tube centrifuged again and the supernatant discarded. The bacterial cell pellet was resuspended in 250 µL of Buffer P1. Buffer P2 (250 µL) was then added and the tube inverted several times before addition of 350 µL of Buffer N3. After 5 min incubation at room temperature, the tube was centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a Qiagen Spin Column that was centrifuged for 60 sec at 13,000 rpm and the eluent discarded. The column was washed with 500 µL of Buffer PB and then 750 µL of Buffer PE, and the eluent discarded after each centrifugation. To elute plasmid DNA, 50 µL of Buffer EB was placed in the spin column that was incubated at room temperature for 5 min before centrifuging for 1 min into a clean microfuge tube. The plasmid DNA solution was then stored at -20°C.

#### *2.1.3.2 Large scale preparation of plasmid DNA and purification by CsCl-gradient*

Plasmid DNA for injection into rabbits was purified by two rounds of CsCl gradient ultracentrifugation as described in Sambrook and Russell (2001). To prepare plasmid DNA, 1 L of *E. coli* DH5α culture containing the vaccine plasmid (Table 2.1) was centrifuged in 500 mL Dryspin bottles (Nalgene, USA) at 5000 rpm in an SLA-3000 rotor (Sorvall RC5+ centrifuge) to collect bacteria. The medium was poured off, the pellet resuspended in 200 mL of STE buffer (100 mM NaCl, 100 mM Tris.HCl [Progen, Australia], 10 mM EDTA [BDH, Australia], pH 8.0), and then recentrifuged. After pouring off the supernatant, the pellet was resuspended in 72 mL of ice-cold Solution I (50 mM glucose, 25 mM Tris.HCl, 10 mM EDTA, pH 8.0), then 160 mL of Solution II (200 mM NaOH [BDH, Australia], 1% SDS [BDH, Australia]) was added. The tube was inverted several times to thoroughly mix the bacteria with Solution II, then incubated at room temperature for 10 min. Ice-cold Solution III (40mL; 3 M potassium acetate [Ajax,



Australia], 11.5% glacial acetic acid [BDH, Australia]) was then added, and the mixture gently shaken. After incubating on ice for a further 10 min, the precipitate was removed by centrifugation at 5000 rpm in an SLA 3000 rotor for 20 min. The supernatant was poured off into a clean 500 mL Dryspin bottle, ensuring no precipitate followed. Isopropanol (105 mL; BDH, Australia) was added to precipitate plasmid DNA, and the mixture incubated at room temperature for 15 min. Plasmid was pelleted by centrifugation (6000 rpm for 20 min) at 25°C. The pellet was washed twice with 100mL of 70% ethanol (BDH, Australia) and allowed to dry in air. The plasmid DNA was then dissolved in 6 mL of TE buffer (2 mM Tris.HCl, 5 mM EDTA, pH 8.0).

To further purify the plasmid DNA and remove any endotoxin, caesium chloride (CsCl) gradient ultracentrifugation was performed. Briefly, 1 g of CsCl (Sigma, Australia) was added per millilitre of plasmid DNA solution. This resulted in a final CsCl concentration of approximately 0.8 g/mL. Ethidium bromide solution (10 mg/mL; Sigma, Australia) was added at 800  $\mu$ L per 10 mL of plasmid/CsCl solution. Beckman 342413 Quickseal ultracentrifuge tubes (13.5 mL) were filled with the resulting solution. The tubes were sealed and centrifuged in an 80Ti rotor at 60,000 rpm for 21 h. The plasmid DNA band was removed using an 18G needle and syringe, and diluted with TE buffer containing 1 g/mL of CsCl to a final volume of 13.5 mL. The ultracentrifugation step was repeated, and the plasmid DNA band again removed. Ethidium bromide was extracted using water-saturated butanol (BDH, Australia), and CsCl removed by at least three rounds of dialysis against TE buffer, until there was no butanol odour in the dialysis buffer. The purified plasmid DNA solution was stored at -20°C.

### **2.1.4 Determination of DNA concentration**

The concentration of DNA in a solution was measured using spectrophotometry. The DNA solution was diluted 1:200 with MilliQ water, and the absorbance at 260 nm measured using a Varian Cary Bio UV-Visible Spectrophotometer. The concentration was calculated by multiplying the absorbance by the dilution factor (200) and by the absorptivity of double-stranded DNA ( $50 \text{ mL.cm}^{-1} \mu\text{g}^{-1}$ ) to obtain a DNA concentration in  $\mu\text{g/mL}$ .

### **2.1.5 Agarose gel electrophoresis**

Agarose gel electrophoresis was performed using 0.6-1.5% agarose (Progen, Australia) in 1X TAE buffer (40 mM Tris.HCl, 0.9 mM EDTA, pH adjusted to 8.0 with glacial acetic acid). Agarose gel loading buffer (1/6 of sample volume; 25% glycerol, 5.6  $\mu\text{g/mL}$  bromophenol blue [Sigma, Australia]) was added to samples before loading onto the gel. The electrophoresis was carried out with 1X TAE buffer as the running buffer at a constant voltage of 90 V until the blue dye front was about 80% down the gel. After electrophoresis, the gel was stained for 20 min in 1  $\mu\text{g/mL}$  ethidium bromide solution, then destained in MilliQ water for 30 min. DNA bands were visualised using a UV transilluminator, and recorded using a CCD camera and NIH Image software.

### **2.1.6 Purification of DNA from agarose gels**

To purify DNA after gel electrophoresis (plasmids, PCR products and restriction fragments), the band containing the DNA of interest was excised from the gel and transferred to a microfuge tube. The DNA was purified using the Qiagen Gel Extraction Kit, according to the manufacturer's instructions. Briefly, the gel slice was weighed and three volumes of Buffer QC added. The tube was incubated at 50°C until the gel had dissolved completely. One gel volume of isopropanol was added to the tube and the

mixture vortexed. The mixture was then transferred to a QIAquick spin column and centrifuged at 13,000 rpm for 1 min and the eluent discarded. The column was washed with 750  $\mu$ L of Buffer PE and the eluent discarded. To elute the DNA, 30  $\mu$ L of Buffer EB was added to the column that was incubated at room temperature for 5 min before centrifuging at 13,000 rpm for 1 min into a clean microfuge tube. The DNA solution was stored at -20°C until required.

## **2.1.7 Northern analysis**

### *2.1.7.1 RNA gel electrophoresis and blotting*

To detect the presence of RNA transcripts, Northern blot analysis was used. RNA from cells was prepared as described in Section 2.4.4. A 1.1% agarose/formaldehyde gel was prepared by dissolving 0.55 g of agarose in 36 mL of diethylpyrocarbonate (DEPC; Sigma, Australia)-treated MilliQ water and cooling to 60°C. RNA gel buffer (5 mL of a 10X solution; 200 mM triethanolamine [BDH, Australia], 50 mM EDTA, pH 7.4) was added with 9 mL of 36% formaldehyde (BDH, Australia), giving a final concentration of 2 M formaldehyde. The gel was allowed to set in an RNase-free electrophoresis tank and covered with RNA running buffer (20 mM triethanolamine, 5 mM EDTA, 1.8 M formaldehyde, pH 7.4). RNA samples were prepared by adding 6  $\mu$ L of RNA to 14.4  $\mu$ L of RNA sample buffer (66% deionised formamide [BDH, Australia], 2.2 M formaldehyde, 27 mM triethanolamine, 6.7 mM EDTA, pH 7.4) and denaturing at 60°C for 15 min. After adding 2  $\mu$ L of 6X agarose gel loading buffer, the RNA samples were loaded onto the gel and electrophoresed at a constant voltage of 90 V for 2 h. The gel was stained in 1  $\mu$ g/mL ethidium bromide solution for 20 min, destained in DEPC-treated water for 2-3 h and visualised as described in Section 2.1.5. The gel was then submerged in 20X SSC (300 mM NaCl, 3 M citric acid [BDH, Australia]) for 30 min and then RNA transferred onto a

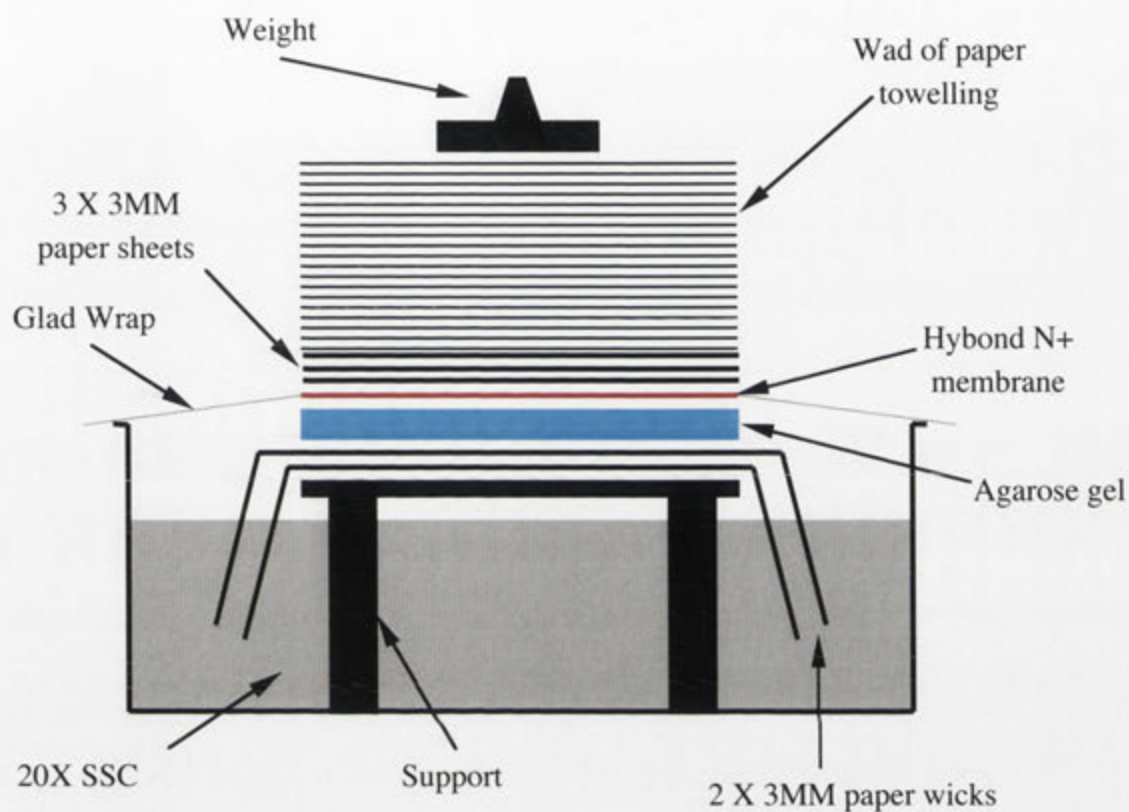
Hybond N<sup>+</sup> membrane (Amersham, USA) using the apparatus shown in Figure 2.1. Following overnight transfer at room temperature, the membrane was removed and baked at 120°C for 2 h to bind the RNA to the membrane.

#### *2.1.7.2 Probe preparation*

The membrane was probed with digoxigenin (DIG)-labelled DNA prepared using the DIG High-Prime DNA Labelling and Detection Kit (Roche, Germany) according to the manufacturer's instructions. The probe DNA required for each Northern blot corresponded to the gene whose transcripts were under investigation. This DNA was purified by gel electrophoresis following restriction endonuclease reaction or PCR amplification, and purified as described in Section 2.1.6. For the labelling reaction, 1 µg of probe DNA was suspended in 16 µL of DEPC-treated water and boiled for 10 min to denature the DNA. The tube was then placed on ice and 4 µL of DIG-High Prime Solution added to the probe DNA solution. The reaction was vortexed briefly, incubated at 37°C for 3-5 h, and then boiled for 10 min before placing on ice. The labelled probe solution was then ready to be added to the hybridisation reaction.

#### *2.1.7.3 Membrane hybridisation*

The fixed nylon membrane carrying the RNA was prehybridised in a Hybaid bottle with 50 mL of high-SDS hybridisation solution (50% deionised formamide, 50 mM Na<sub>3</sub>PO<sub>4</sub> [BDH, Australia], 7% SDS, 0.1% N-laurylsarcosine [Sigma, Australia], 10% Blocking Solution [DIG High-Prime Kit, Roche, Germany], 750 mM sodium citrate, 75 mM NaCl, pH 7.0) for 2 h at 65°C. After prehybridisation, the denatured, labelled probe DNA (Section 2.1.7.2) was added and the tube incubated overnight at 65°C. In the morning, the hybridisation solution was poured off and the membrane washed at 70°C for 15 min, twice



**Figure 2.1** – Construction of the apparatus for the transfer of RNA onto a nylon membrane.

with 50 mL of 2X wash solution (300 mM sodium citrate, 30 mM NaCl, 0.1% SDS, pH 7.0), twice with 50 mL of 0.5X wash solution (75 mM sodium citrate, 7.5 mM NaCl, 0.1% SDS, pH 7.0) and once with 0.2X wash solution (30 mM sodium citrate, 3 mM NaCl, 0.1% SDS, pH 7.0). The membrane was then removed from the Hybaid bottle and washed once with maleic acid buffer (100 mM maleic acid [Ajax, Australia], 150 mM NaCl, pH 7.5).

#### *2.1.7.4 Visualisation of probe binding*

After washing, the membrane was probed with a monoclonal antibody against DIG, and binding of the antibody was detected by enhanced chemiluminescence (ECL). All steps were performed at room temperature using the DIG High-Prime DNA Labelling and Visualisation Kit (Roche, Germany) according to the manufacturer's instructions. Briefly, the membrane was transferred to a hybridisation bag and blocked with 20 mL of antibody blocking solution (90 mM maleic acid, 135 mM NaCl, 10% Blocking Solution [Roche, Germany], pH 7.5) for 30 min. The antibody blocking solution was poured off and replaced with 20 mL of antibody blocking solution containing 2  $\mu$ L of alkaline phosphatase-conjugated anti-DIG antibody (Roche, Germany). After incubating for 30 min, the membrane was washed twice with 20 mL of antibody wash buffer (100 mM maleic acid, 150 mM NaCl, 0.3% Tween 20 [Sigma, Australia], pH 7.5), 15 min each wash. Finally, all buffer was removed, the membrane covered with CSPD Ready-to-Use Reagent (Roche, Germany), sealed in a clean hybridisation bag and exposed to Hyperfilm ECL (Amersham, USA), which was developed using a Kodak X-OMAT automated x-ray film processor.

## 2.1.8 Polymerase Chain Reaction (PCR) methods

### 2.1.8.1 Standard PCR

PCR was used for several purposes in this study. Oligonucleotide primers (Tables 2.2 and 2.3) were designed based on published sequences obtained from Genbank databases to amplify specific sequences under investigation. Primers were commercially manufactured by Proligo Australia as a stock solution of 100 mM in water. Primers were diluted to a working concentration of 10 mM with MilliQ water. The standard PCR method used the following reaction mix, time and conditions:

- 1  $\mu$ L of forward primer (10 mM stock solution)
- 1  $\mu$ L of reverse primer (10 mM stock solution)
- 2  $\mu$ L 10X *Taq* DNA polymerase buffer (Fischer Biotech, USA)
- 1.5  $\mu$ L  $MgCl_2$  (25 mM stock solution; Fischer Biotech, USA)
- 1.5  $\mu$ L dNTPs (10 mM each stock solution; Fischer Biotech, USA)
- 1 unit of *Taq* DNA polymerase (Fischer Biotech, USA)
- Template DNA (generally 2-5  $\mu$ L)
- sterile MilliQ water to 20 $\mu$ L

Following careful mixing of the reaction, it was placed in a capillary tube in a Corbett Research FTS-IS Capillary Thermal Sequencer and subjected to the following temperature program:

- |         |  |
|---------|--|
| 1 X     | rapid ramp to 95°C for 2.5 min                           |
| 30-45 X | rapid ramp to 95°C for 30 sec                            |
|         | rapid ramp to annealing temperature (50-60°C) for 30 sec |
|         | rapid ramp to 72°C for 1-4 min (extension)               |
| 1 X     | 72°C for 4 min.  |

**Table 2.2 - PCR primers used in the amplification and cloning of myxoma virus genes and rabbit cytokines.** The restriction sites incorporated in the primers are underlined, and the start or stop codons included in the primer are in bold.

**M055R Fwd (*EcoRI*)**

5' TTG GAA TTC **ATG** GGT GCC GCC GCG AG 3'

**M055R Rev (*XhoI*)**

5' CGT CTC GAG CAG TTT **TTA** AAC ACT GTG TAC C 3'

**M073R Fwd (*EcoRI*)**

5' CGT GAA TTC **ATG** TCT TGG TCC ATA AAC CTA G 3'

**M073R Rev (*XhoI*)**

5' CTG CTC GAG GAG **TTA** TTT TTT GGT TGT CTG 3'

**M107L Fwd (*EcoRI*)**

5' CTG GAA TTC **ATG** AGT TAT TTA AGT TAT TAC AAT ATG 3'

**M107L Rev (*XhoI*)**

5' CGT CTC GAG ATA ATT ATT **AAT** CTT CGT AGA AGC 3'

**M115L Fwd (*EcoRI*)**

5' CGT GAA TTC **ATG** GAT AGA GTG TTG TCG ATA TAC 3'

**M115L Rev (*XhoI*)**

5' CGT CTC GAG GTT TCT GTA ACG **ATT** ATA GAG C 3'

**M121R Fwd (*EcoRI*)**

5' CGT GAA TTC **ATG** TCG TTA CAC GCC AAC G 5'

**M121R Rev (*XhoI*)**

5' CGT CTC GAG TTT TCA TTT ATA **ACT** ATA AAG TAA ATG 3'

**IL2 Fwd (*NheI*)**

5' CGT GCT AGC **ATG** TAC AAA GTA CAA CTC TTG 3'

**IL2 Rev (*HindIII*)**

5' CGT AAG CTT **TTA** TGA ACT CGA TGC TCA G 3'

**IL4 Fwd (*NheI*)**

5' CGT GCT AGC **ATG** GGG CTC CCT GCC 3'

**IL4 Rev (*HindIII*)**

5' CTG AAG CTT **TCA** GCT CTG ACG CTT TG 3'



**Table 2.3 – Primers used in the construction and screening of recombinant myxoma viruses.** Restriction sites incorporated into the primers are underlined.

**M007L-R2** (*XhoI*)

TCCTCGAGACGTTCAAACATGGAG

**M007-R1**

TCCGTAGACGGACACTTCC

**M007L-F2** (*HindIII*)

CGAAGCTTAAACGAGCGGAGTACGTC

**M007L-R1** (*EcoRI*)

AAGAATTCTACGCTGGTACCTGTTC

**M007L-F1** (*BamHI*)

GTGGATCCTCGCGTCGCTCGCTATC

**M008L-F1** (*BamHI*)

ACGGATCCTGTCACTTCGCCACCATG

**M009L-R1** (*XhoI*)

GACTCGAGCGTCGCTCATTGCACCATC

**M010L-F1** (*HindIII*)

CTACGTGGGAAGCTTATGTCAG

**M010L-R1**

TAGAATTCCAACGAAGGCATCGTTGC

**M011L-R1** (*EcoRI*)

CTATGAATTCCGAGAACATAGC

**M012L-F1** (*BamHI*)

ACGGATCCATAACGGATTTCGGTTCAAG

**M062R-R1** (*BamHI*)

CAGGATCCGTGATCAACCTGTTCAG

**M063R-R1** (*EcoRI*)

GTGAATTCGCAACTGTGCCCCGTGAC

**M063R-F1**

GAGAAGGAGTACTGCTTCAC

**M063R-F2** (*HindIII*)

CGAAGCTTATACCAGCAGTGAGGAAG

**M064R-R1** (*XhoI*)

CCCTCGAGATAACGACTTTATAAATAAGTG

**gpt-R1**

CAACATGTCCCAGGTGACG

**gus-F1**

GGACTGGCATGAACTTCG

If not analysed immediately by agarose gel electrophoresis (Section 2.1.5), PCR products were stored at -20°C.

#### 2.1.8.2 Colony PCR

Colony PCR was used to screen transformed *E. coli* colonies for the presence of the required insert DNA. Template DNA was prepared by suspending a colony of bacteria in 50 µL of MilliQ water, and incubating at room temperature for 10 min. After vortexing vigorously, 5 µL of this suspension was used immediately in a PCR as described in Section 2.1.8.1, and the remainder stored at 4°C. If the colony was found to contain the required insert DNA, the colony preparation was restreaked from the 50 µL aliquot onto an LB agar plate supplemented with the appropriate antibiotic and incubated at 37°C overnight.

#### 2.1.9 DNA Sequencing

DNA was sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, USA). To 4 µL of the Ready Reaction Mixture was added 300 ng of template DNA, 3.75 pmol of sequencing primer and MilliQ water to a final volume of 10 µL. The reaction was subjected to the following temperature program in a Corbett Research FTS-IS Capillary Thermal Sequencer:

25 cycles of [95°C for 10 sec; 50°C for 5 sec; 60°C for 4 min]

Once completed, excess dye terminators were removed by adding water (10 µL), 2 µL of 3 M sodium acetate [BDH, Australia] and 50 µL of ethanol. The mixture was placed on ice for 20 min, followed by 20 min of centrifugation at 13,000 rpm. The supernatant was removed and the DNA pellet washed three times with 500 µL of ice-cold 70% ethanol. The tube was centrifuged for 5 min and the wash solution removed. The pellet was then air-dried. Once dried, the reactions were electrophoresed and the sequence recorded using an

ABI 3730 Sequencer (Applied Biosystems, Australia) at the John Curtin School of Medical Research Biomolecular Resources Facility at the Australian National University.

## **2.2 SDS-PAGE AND IMMUNOBLOTTING**

### **2.2.1 Preparation and electrophoresis of SDS-PAGE gels**

During this project, size-separation of proteins was performed prior to immunoblotting or other forms of analysis. This was accomplished using the BioRad Mini Protean II system. All experiments used 1 mm thick, 12% SDS-PAGE resolving gels topped with a 4.7% stacking gel and were prepared in duplicate. The resolving gel solution was prepared by mixing 1.5 mL of 40% acrylamide solution (29 parts acrylamide, 1 part bis-acrylamide; Amresco, USA), 2.25 mL of MilliQ water and 1.25 mL of resolving gel buffer (500 mM Tris.HCl, 0.4% SDS, pH 6.8). The gel was polymerised by adding 5 µL of N,N,N',N'-tetramethylethylenediamine (TEMED; BioRad, Australia) and 50 µL of a 10% ammonium persulfate solution (APS; Biorad, Australia), and the solution immediately transferred to the gel housing for setting. Once the resolving gel had set, a stacking gel was prepared by mixing 625 µL of stacking gel buffer (1.5 M Tris.HCl, 0.4% SDS, pH 8.8), 300 µL of 40% acrylamide and 1.63 mL of MilliQ water. TEMED (5 µL) and 10% APS (50 µL) were added and solution layered on top of the resolving gel. A teflon comb with the required number of wells was immediately inserted and the stacking gel was set at room temperature for 30 min. Once set, the comb was removed, the gels were placed into the gel tank and covered with SDS-PAGE running buffer (0.1% SDS, 24 mM Tris.HCl, 192 mM glycine [BDH, Australia]).

Samples were prepared by mixing equal volumes of protein sample and 2X sample loading buffer (SLB; 62.5 mM Tris.HCl, 10% glycerol, 2% SDS, 0.4 mg/mL bromophenol blue,

pH 6.8). If denaturing conditions were required, 2X SLB was supplemented with dithiothreitol (DTT; Sigma, Australia) to a final concentration of 100 mM. Samples were then boiled for 3 min and cooled on ice. Samples (20  $\mu$ L) were added to each well and electrophoresed at a constant 100 V for 90 min. Protein molecular weight markers (Benchmark Pre-Stained Markers [GibcoBRL, USA]) were run on each gel, to allow determination of the molecular weight of proteins under analysis. After electrophoresis, the gels were removed from the apparatus and the stacking gel removed and discarded.

### **2.2.2 Coomassie blue staining of SDS-PAGE gels**

To visualise proteins in the SDS-PAGE gels, the resolving gel was placed in 50 mL of Coomassie Blue staining solution (45% methanol, 9% glacial acetic acid, 1.8 g/L Coomassie brilliant blue [Sigma, Australia]) and gently agitated for 60 min on a rocking table. The gel was then removed and destained in 100 mL of destaining solution (23% ethanol, 9.6% glacial acetic acid) for 60 min. The destaining solution was replaced and the gel destained overnight. Destaining solution replacement was continued until the background of the gel was completely destained.

### **2.2.3 Silver staining of SDS-PAGE gels**

Silver staining was performed using the Bio-Rad Silver Stain Kit according to the manufacturer's instructions. After electrophoresis, the gel was removed from the apparatus, placed in 100 mL of Fixative-1 (40% methanol, 10% glacial acetic acid) and gently agitated for 30 min. The gel was then transferred to 100 mL of Fixative-2 (40% methanol, 5% glacial acetic acid) for 15 min, and then to a fresh aliquot of Fixative-2 for 15 min. The gel was then placed in 50 mL of Oxidiser Solution (Biorad, Australia), gently agitated for 5 min, then washed twice in 100 mL of MilliQ water. The gel was transferred

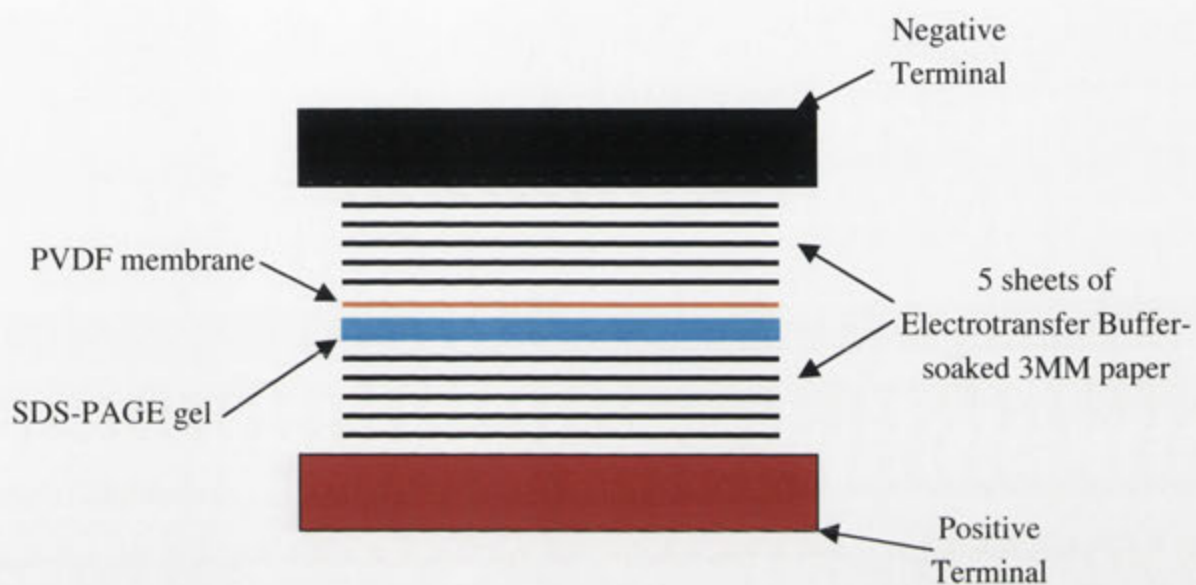
to 50 mL of Silver Reagent (10% Silver Reagent Concentrate in MilliQ water [Biorad, Australia]) for 20 min with shaking, and then washed in 100 mL of MilliQ water for 1 min. Developer Solution (50 mL; 32 g/L Developer Powder [Biorad, Australia]) was added to the gel until a smoky precipitate formed (approximately 1 min), when it was replaced with a further 50 mL of Developer Solution. The gel was left in the Developer Solution until bands were clearly visible, at which point the gel was placed in Stop Solution (5% glacial acetic acid) for 5 min.

#### **2.2.4 Transfer of proteins from SDS-PAGE gels to PVDF membranes (immunoblotting)**

To perform immunoblot analysis, proteins were transferred from the SDS-PAGE gel to PVDF membranes using the Hoefer SemiPhor transfer apparatus. After electrophoresis, gels were placed in Electrotransfer Buffer (25 mM Tris.HCl, 192 mM glycine, 0.1% SDS, 15% methanol) for 20 min. Ten sheets of 3MM paper of the same size as the gel were soaked in Electrotransfer Buffer. An Immobilon-P PVDF membrane (Millipore, Australia) of the same size as the gel was wet with methanol, and then soaked in Electrotransfer Buffer for 5 min. The apparatus was assembled as shown in Figure 2.2. The proteins were transferred by applying a constant current of 0.8 mA per cm<sup>2</sup> of gel area for 45 min. After transfer, the membrane was removed and washed in Tris-buffered saline (TBS; 137 mM NaCl, 25 mM Tris.HCl, 2.7 mM KCl [Ajax, Australia], pH 8.0). The membrane was then used in immunostaining immediately, or blotted dry and used within a few days.

#### **2.2.5 Immunostaining of membranes**

PVDF membranes were probed with antibodies for the presence of specific proteins. Membranes were first blocked using 50 mL of blocking buffer (10% skim milk powder



**Figure 2.2** – The arrangement of the Hoefer SemiPhor transfer system for the transfer of proteins separated on an SDS-PAGE gel to a PVDF membrane.

[Diploma, Australia] in TBS) overnight at 4°C, or for 1 h at room temperature. The membrane was then washed three times in 50 mL of TTBS (0.1% Tween 20 in TBS) with agitation at room temperature. The primary antibody was then diluted in 20 mL of antibody buffer (2% skim milk powder, 0.05% Tween 20 in TBS) to the required dilution factor (1/100 to 1/5000), and incubated with the membrane with agitation at room temperature for 2 h. The membrane was then washed three times with 50 mL TTBS with agitation. The secondary antibody conjugated to horseradish peroxidase (HRP; Silenus Labs, Australia) was then diluted 1/1000 with 20 mL of antibody buffer and incubated at room temperature with the membrane for 1 h. The membrane was then washed twice with 50 mL of TTBS with agitation, and then once with 50 mL of TBS. The membrane was now ready to visualise specific binding of the primary antibody.

### **2.2.6 Visualisation of antibody binding**

Chemiluminescent detection of specific antibody binding to the membrane was done using the ECL Western Blotting Analysis System (Amersham Biosciences, United Kingdom), according to the manufacturer's instructions. Equal volumes of the two kit reagents (Detection Reagents 1 and 2) were mixed and allowed to reach room temperature. Enough of the reagents were prepared such that there was 0.125 mL/cm<sup>2</sup> of membrane. The solution was placed on the immunostained membrane (Section 2.2.5) such that the entire surface was in contact with the Detection Reagent and incubated at room temperature for 1 min. Excess reagent was poured off, and the membrane wrapped in plastic film and placed into an autoradiography cassette. The membrane was exposed to Hyperfilm ECL which was developed using a Kodak X-OMAT automated x-ray film processor.

## **2.3 PREPARATION OF RECOMBINANT MYXOMA VIRUS ANTIGENS AS 6XHis-TAGGED FUSION PROTEINS**

Recombinant proteins were produced in an *E. coli*-based pET system (Novagen, Germany), utilising the T7-*lac* promoter system for high production levels of recombinant proteins (Novagen, 2003; Studier *et al.*, 1990). Proteins were purified using TALON Metal Affinity Resin (Clontech, USA). These recombinant proteins were used in Western blot analysis and lymphocyte proliferation assays described in Section 2.7.

### **2.3.1 Preparation of producer strain of bacteria and production of protein**

Myxoma virus genes were amplified by PCR (Section 2.1.8) using primers described in Table 2.2. The primers contained an *Eco*RI site immediately before the start codon, and a *Xho*I site a few bases downstream of the stop codon. Purified PCR amplified fragments of myxoma virus open reading frames were digested with *Eco*RI and *Xho*I, and were inserted into the pET30a+ vector (Novagen, Germany) between the *Eco*RI and *Xho*I sites, in frame with the 5' purification tag sequences. The recombinant plasmids were transfected into *E. coli* BL21(DE3), a producer strain of bacteria containing a lysogenic T7-type bacteriophage, DE3. Bacteria were grown overnight at 37°C in LB-kanamycin broth, sub-cultured 1:50 into fresh LB-kanamycin broth and incubated with shaking for 2.5 h at 37°C. Inducer (IPTG to a final concentration of 1 mM) was added and shaking at 37°C continued for a further 3 h.

### **2.3.2 Harvesting of soluble protein from culture**

Cells were collected by centrifugation in 40 mL Oak Ridge Tubes at 5000 rpm in a SS-34 rotor for 20 minutes (Sorvall RC5+ centrifuge), and media poured off. The cell pellet was resuspended in Extraction/Wash Buffer (50 mM Na<sub>3</sub>PO<sub>4</sub>, 300 mM NaCl) such that the



bacteria were concentrated 10-fold from the original culture. Lysozyme (final concentration of 100 µg/mL; Sigma, Australia) was added and the bacteria incubated on ice for 30 min with occasional shaking. The cells were then lysed by sonication on ice (4 pulses of 15 sec, maximum power). The bacterial cell lysate was then centrifuged in 40 mL Oak Ridge Tubes at 10,000 rpm in an SS-34 rotor at 4°C for 30 min (Sorvall RC5C+ centrifuge) to pellet insoluble material. The clarified supernatant was transferred to a clean tube and was used immediately, or stored at -80°C overnight.

### **2.3.3 Purification of recombinant protein on TALON-IMAC resin**

Recombinant protein was purified on TALON IMAC resin (Clontech, USA). A gravity flow column containing 5 mL of TALON resin was washed with 25 mL of Extraction/Wash Buffer. Clarified cell lysate (20 mL) was added to the column and allowed to flow through. The resin was then washed with 25 mL of Extraction/Wash buffer, followed by 25 mL of Imidazole Wash Buffer (50 mM Na<sub>3</sub>PO<sub>4</sub>, 300 mM NaCl, 15 mM imidazole [Sigma, Australia]) to remove non-specifically bound proteins. To elute recombinant proteins, 25 mL of Extraction Buffer (50 mM Na<sub>3</sub>PO<sub>4</sub>, 300 mM NaCl, 150 mM imidazole) was added to the column, and 1 mL fractions of flow-through eluent collected and stored at -80°C.

Individual fractions were analysed by SDS-PAGE. Fractions containing the recombinant protein were pooled and protein precipitated by the addition of 1/10 volume of 1 g/mL trichloroacetic acid (BDH, Australia). The tubes were incubated on ice for 20 min and then centrifuged at 13,000 rpm for 15 min at 4°C (Eppendorf 5415C centrifuge). The protein pellet was washed three times with acetone and then air-dried. Recombinant proteins were

resuspended in phosphate buffered saline (PBS; 137 mM NaCl, 1.76 mM  $\text{KH}_2\text{PO}_4$ , 10.2 mM  $\text{Na}_2\text{HPO}_4$ , 3 mM KCl, pH 7.4) and stored at  $-80^\circ\text{C}$ .

A negative control antigen was prepared as follows: *E. coli* BL21(DE3) containing the pET30a+ vector only was subjected to the same treatment and protein purified. The resulting 10 kDa protein contained all of the N-terminal purification tags, and bacterial products found in the antigen preparations, but no myxoma virus-specific sequences.

## 2.4. CELL CULTURE METHODS

### 2.4.1 Maintenance and sub-culturing of RK-13, Vero, CV-1 and BSC-1 cell lines

RK-13 and Vero cells (obtained from laboratory stocks), and CV-1 and BSC-1 cells (obtained from Ron Jackson, CSIRO Sustainable Ecosystems) were maintained in Minimal Essential Medium (MEM; Gibco, USA), supplemented with 10% foetal calf serum (Gibco, USA), 2 mM L-glutamine (Gibco, USA), 500  $\mu\text{g}/\text{mL}$  amphotericin B (Gibco, USA), 60  $\mu\text{g}/\text{mL}$  penicillin (CSL, Australia) and 100  $\mu\text{g}/\text{mL}$  streptomycin (Sigma, Australia). Medium with all of the above additives will be called MEM(Complete). MEM(Complete) without foetal calf serum will be called  $\text{MEM}(\text{F}^-\text{A}^+)$ . MEM(Complete) without antibiotics (includes penicillin, streptomycin and amphotericin) will be called  $\text{MEM}(\text{F}^+\text{A}^-)$ . MEM(Complete) without foetal calf serum or antibiotics will be called  $\text{MEM}(\text{F}^-\text{A}^-)$ . Each of these media was required for specific protocols.

Cells were sub-cultured every 3-4 days. For a confluent cell monolayer in a T180 cell culture flask (Nunc, USA), the monolayer was washed twice with 20 mL of trypsin diluent (140 mM NaCl, 5 mM KCl, 400  $\mu\text{M}$   $\text{Na}_2\text{HPO}_4$ , 400  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ , 6 mM  $\text{NaHCO}_3$ , 300  $\mu\text{M}$   $\text{Na}_3\text{EDTA}$ , pH 7.3). Trypsin (15 mL of 0.1% solution in trypsin diluent; Gibco, USA) was

added to the flask and incubated at 37°C for 3-5 min. The cells were dislodged and poured into a tube containing 20 mL of MEM(Complete) and the concentration of cells determined by counting with a haemocytometer. Approximately 3 mL of the cells were transferred to a fresh T180 flask with 40 mL of MEM(Complete) and incubated for 3-4 days at 37°C before sub-culturing again. These cells could also be plated out into 6-well, 24-well or 96-well plates as required. Unless otherwise indicated, all incubations during cell culture were performed at 37°C in a humidified, water-jacketed incubator in an atmosphere of 5% CO<sub>2</sub>/95% air.

#### **2.4.2 Transfection of RK13 and Vero cells**

Twenty-four (24)-well plates were seeded with  $1.5 \times 10^5$  cells in 1 mL of MEM(F<sup>+</sup>A<sup>-</sup>) per well. These were grown to 90-95% confluency. Monolayers were washed twice with MEM(F<sup>-</sup>A<sup>-</sup>) and then 500 µL of MEM(F<sup>+</sup>A<sup>-</sup>) was added to each well. In a microfuge tube, 50 µL of MEM (F<sup>-</sup>A<sup>-</sup>) and 3 µL of Lipofectamine 2000 (Gibco Life Technologies, USA) were mixed by gently flicking the tube. This was incubated at room temperature for 5 min and then added to 50 µL of MEM(F<sup>-</sup>A<sup>-</sup>) containing 2 µg of the plasmid to be transfected into cells, and mixed by gently flicking the tube. This mixture was then incubated at room temperature for 20 min and added to 1 well of washed cells. Cells were then incubated at 37°C until required (usually 24 or 48 h).

#### **2.4.3 Protein extraction from plasmid-transfected RK13 cells**

Transfected monolayers were washed twice with 1 mL of PBS, and then 150 µL of 1X SDS-PAGE sample loading buffer was added to each well. All cellular material was scraped from the well using a pipette tip, and the contents transferred to a microfuge tube.

Samples for SDS-PAGE analysis were boiled for 10 min with 15  $\mu\text{L}$  of 1 M DTT (reducing conditions) or with no additional DTT (non-reducing conditions).

#### **2.4.4 RNA Extraction from plasmid-transfected RK13 cells**

Twenty-four hours following transfection, the media was removed, and the monolayer washed with 1 mL of PBS. To each well was then added 200  $\mu\text{L}$  of RNAzol B (Tel-Test, USA). All cellular material was scraped from the well using a pipette tip, and the solution transferred to a 1.5 mL microfuge tube. Chloroform (20  $\mu\text{L}$ ) was added and the tube shaken vigorously for 15 sec. The tube was placed on ice for 5 min and then centrifuged at 13,000 rpm (Eppendorf 5415 D centrifuge) at 5°C for 15 min. The top (aqueous) layer was then removed to a fresh microfuge tube containing 150  $\mu\text{L}$  of isopropanol. This was mixed and placed on ice for 15 min to precipitate the RNA. The tube was centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatant removed. The RNA pellet was washed with 1 mL of 75% ethanol by vortexing briefly, and then centrifuging at 13,000 rpm for 5 min. After removing the supernatant, the pellet was dried briefly under vacuum at room temperature. The pellet was resuspended in 20  $\mu\text{L}$  of DEPC-treated water with 1 U/ $\mu\text{L}$  of DNase (Sigma, Australia), and stored at –80°C.

#### **2.4.5 Immunofluorescent detection of protein production in transfected cells**

After transfection, cells were incubated for 24 h. Monolayers were washed twice with 500  $\mu\text{L}$  of PBS, then fixed with 250  $\mu\text{L}$  of methanol:acetone (1:1) for 10 min. After removing the methanol:acetone, monolayers were then washed twice with PBS. Polyclonal anti-myxoma virus serum from a hyperimmune rabbit was utilised to detect myxoma virus antigens. Serum was diluted 1:400 with PBS containing 3% bovine serum albumin (BSA; Amersham, UK) and incubated with cell monolayers for 90 min. Monolayers were washed

twice with PBS containing 3% BSA and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit Ig (Silenus Labs, Australia) for 60 min. After washing twice with PBS, specific binding of antibody was observed using a Leitz Fluovolt FS microscope and recorded with a Spot RT digital camera (Diagnostic Instruments, Australia).

#### **2.4.6 *In vitro* confirmation of rabbit interleukin-4 expression**

Expression of rabbit interleukin-4 (IL-4) was detected using immunofluorescence. RK13 monolayers transfected with IL-4-expressing plasmids were fixed as described in Section 2.4.5. Anti-rabbit IL-4 antiserum had previously been raised in rats immunised with a synthetic peptide based on Helix A of rabbit IL-4. This serum binds specifically to rabbit IL-4 on both immunoblots and transfected cells (Kerr *et al.*, 2004). The serum was diluted 1/100 in PBS containing 3% BSA, added to fixed monolayers and incubated at room temperature for 90 min. Antibody binding was detected using FITC-conjugated anti-rat IgG secondary antibody (Silenus Labs, Australia) and observed as described in Section 2.4.5.

#### **2.4.7 Concentration of supernatants from tissue culture cells**

For the analysis of proteins secreted from transfected or virus-infected cells, supernatants were concentrated. This was achieved using Centricon Centrifugal Filter Devices (Amicon Bioseparations, USA) according to the manufacturer's instructions. After 24-48 h incubation, supernatants from transfected or infected RK13 cell monolayers were collected without disturbing the cell monolayer. Supernatant (2 mL) was placed into the sample reservoir of a Centricon YM-10 tube and the device assembled according to the manufacturer's instructions. The tubes were centrifuged at 5000 rpm in an SS-34 rotor at 25°C for 1 h. This resulted in the 50-fold concentration of supernatants (retentate volume

of 40  $\mu$ L). The retentate was collected by removing the filtrate vial, capping the sample reservoir and inverting the tube. The tube was centrifuged at 5000 rpm in an SS-34 rotor for 10 min to collect the concentrated retentate. This was stored at  $-80^{\circ}\text{C}$  until required for further analysis.

#### **2.4.8 HT-2 bioassay for *in vitro* confirmation of interleukin-2 expression**

The production of rabbit interleukin-2 (IL-2) from IL-2-expressing plasmid-transfected cells was detected using the mouse lymphocyte cell line HT-2, which has an absolute dependence on IL-2 for proliferation (Wadhwa *et al.*, 1995). Rabbit IL-2 is able to induce proliferation of this cell line (Peter Kerr, personal communication). HT-2 cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 500  $\mu\text{g/mL}$  amphotericin B, 60  $\mu\text{g/mL}$  penicillin, 100  $\mu\text{g/mL}$  streptomycin, 1 mM sodium pyruvate (Sigma, Australia), 10 mM NaCl and 6.2  $\mu\text{L/L}$  monothioglycerol (Sigma, Australia). This media was called RPMI(HT-2). This was supplemented with 0.1% recombinant mouse IL-2 solution produced in Hi5 cells (obtained from Barbara van Leeuwen, School of Biochemistry and Molecular Biology) for the growth and passaging of HT-2 cells. For use in the bioassay, cells were collected by centrifugation at 1200 rpm in a Beckman Allegra 6R centrifuge for 10 min, and the supernatant removed. The cells were washed 3 times with 30 mL of RPMI(H-T2) without supplemental IL-2 to remove residual IL-2. All following steps used media without supplemental IL-2. Cells were then counted using a haemocytometer.

To measure the production of rabbit IL-2 from IL-2-expressing plasmid-transfected RK13 cells, cells were incubated for 48 h following transfection and the supernatant collected and concentrated by a factor of ten (Section 2.4.7). Two-fold serial dilutions of the

concentrated supernatants were performed using RPMI(HT-2), from 0 to 1/1024 and 50  $\mu$ L of each dilution added to quadruplicate wells in round bottom 96-well plates. As a positive control, recombinant mouse IL-2 was diluted 1/100 and then serially diluted as described above. Concentrated supernatants from cells transfected without plasmid (mock-transfected) was used as negative controls. To each well,  $5 \times 10^4$  cells of HT-2 were added in 50  $\mu$ L of RPMI(HT-2). Plates were incubated for 24 h at 37°C in humidified air with 5% CO<sub>2</sub>. To measure IL-2 induced proliferation, 0.5  $\mu$ Ci of <sup>3</sup>H-thymidine (Amersham, UK) was added in 10  $\mu$ L of RPMI(HT-2) to each well and the plates incubated for a further 6 h. The plates were then stored at -80°C. Cells were harvested using a Packard Filtermate 96 onto an EasyTab-C Self-Aligning Glass Filter (Packard, Australia). The glass filter was dried at 37°C for 20 min and loaded into an OmniFilter cassette (Packard, Australia). Microscint O scintillant fluid (20  $\mu$ L; Packard, Australia) was added to each well of the glass filter, and <sup>3</sup>H-thymidine incorporation counted on a Packard Topcount NXT scintillation counter (Packard, Australia).

## **2.5 VIRUS CULTURE METHODS**

### **2.5.1 Virus strains**

The Standard Laboratory Strain of myxoma virus (SLS) used in this study is derived from a freeze-dried rabbit tissue stock prepared by Professor Frank Fenner (John Curtin School of Medical Research, Canberra, Australia) in 1953. This virus is of Grade I virulence (Fenner and Marshall, 1957), killing 100% of infected laboratory rabbits with an average survival time of less than 13 days (Robinson *et al.*, 1999). The Lausanne strain of myxoma virus has been completely sequenced (Cameron *et al.*, 1999) and was used as the template for primer design in the cloning of viral antigen genes (Tables 2.2 and 2.3). The Uriarra strain of myxoma virus was derived by plaque purification by Russell and Robbins (1989) from

the Uriarra/2/53-1 isolate (Mykytowycz, 1953). This virus is of Grade 5 virulence and more than 95% of infected laboratory rabbits recover from infection although they do develop moderate to severe clinical myxomatosis (Fountain *et al.*, 1997; Best and Kerr, 2000). Viruses used in this study were prepared in RK13 cell monolayers that had been infected with testis samples taken from infected laboratory rabbits 8-9 days after infection with the viruses. Virus stocks used were then concentrated (Section 2.5.4) and the titre determined as described in Section 2.5.5.

### **2.5.2 Preparation of viral DNA**

For PCR amplification of viral DNA sequences, myxoma virus DNA was purified using the Qiagen DNAeasy Tissue Kit according to the manufacturer's instructions. Briefly, a T75 flask of cells was infected with virus at a multiplicity of infection (MOI) of 1 and incubated at 37°C for 4-5 days, until complete cytopathic effect (CPE) was observed. The flask was freeze/thawed twice (-80°C to 37°C) and the virus/cell lysate collected into a 40 mL Oak Ridge tube. The tube was centrifuged at 12,000 rpm in an SS-34 rotor for 2 h at 4°C, and the supernatant poured off. The cell pellet was resuspended in 200 µL of PBS and transferred to a microfuge tube. Buffer AL (200 µL) and 20 µL of proteinase K solution was added and the mixture incubated at 70°C for 15 min. Ethanol (200 µL) was added to the tube and then vortexed briefly to ensure mixing. The mixture was transferred to a DNAeasy Spin Column, centrifuged at 8000 rpm for 1 min (Eppendorf 5415C centrifuge) and the eluent discarded. The column was washed with 500 µL of Buffer AW1, and then 500 µL of Buffer AW2. After discarding the eluent, the column was centrifuged at 13,000 rpm for 3 min to remove residual wash buffer. The column was transferred to a clean microfuge tube and 100 µL of Buffer AE added to the column, which was incubated at



room temperature for 5 min. Viral DNA was eluted by centrifugation at 13,000 rpm for 1 min, and the DNA stored at -20°C until required.

### **2.5.3 Preparation of recombinant viruses**

#### *2.5.3.1 Infection of cells with virus and transfection with homologous plasmid*

To prepare deletion mutants of the Uriarra strain of myxoma virus, a monolayer of RK13 cells was grown in a 6-well plate until 90-95% confluent. The monolayer was washed twice with 1 mL of MEM(F<sup>-</sup>A<sup>-</sup>), and then infected with 1x10<sup>6</sup> pfu of Uriarra (MOI=1) in 400 µL of MEM (F<sup>-</sup>A<sup>-</sup>) and incubated at 37°C for 1 h, rocking every 10 min. The virus dilution was removed and the monolayer washed twice with 1 mL of MEM(F<sup>-</sup>A<sup>-</sup>) to remove residual virus, and then 2.5 mL of MEM(F<sup>-</sup>A<sup>-</sup>) added.

Plasmids used for the preparation of recombinant viruses are described in Table 2.1. To prepare the plasmid DNA for transfection, 5 µg of the required plasmid was added to 250 µL of MEM(F<sup>-</sup>A<sup>-</sup>). In a separate tube, 15 µL of Lipofectamine 2000 was added to 250 µL of MEM(F<sup>-</sup>A<sup>-</sup>) and incubated at room temperature for 5 min. The plasmid DNA and Lipofectamine solutions were mixed by flicking the tube and incubated at room temperature for 20 min, then the entire mixture was added to the well containing the Uriarra-infected RK13 monolayer. The cells were incubated at 37°C for 5 h, and the monolayer washed twice with 1 mL of MEM(F<sup>-</sup>A<sup>-</sup>). Finally, 3 mL of MEM(F<sup>-</sup>A<sup>+</sup>) supplemented with 250 µg/mL of xanthine (Sigma, Australia), 10 µg/mL of mycophenolic acid (Sigma, Australia) and 1X HAT supplement (Gibco, USA) was added to the well if using *gpt* selection (this media will be called MEM[Selective]) or 3 mL of MEM(F<sup>-</sup>A<sup>+</sup>) if using *gus* selection, and the cells returned to the 37°C incubator for 2-4 days, until complete CPE was observed on the monolayer. The plate was then freeze/thawed twice to

disrupt cells and release virus, and the cell lysate collected. This was called the recombinant virus preparation, and was stored at -80°C.

#### *2.5.3.2 Purification of recombinant virus using guanine phosphorybosyl transferase (gpt) selection*

To isolate pure recombinant virus, several rounds of plaque purification were performed. The recombinant virus preparation was sonicated using a Branson Sonifier 450 Cup Sonicator (50% Duty, Output 5) for 40 sec. The sonicated preparation was then serially diluted in tenfold steps using MEM(F<sup>-</sup>A<sup>+</sup>) down to 10<sup>-6</sup>. RK13 monolayers in 6-well plates were washed twice with MEM(F<sup>-</sup>A<sup>+</sup>), infected with 400 µL of serially diluted virus (10<sup>-1</sup> to 10<sup>-6</sup> in separate wells) and incubated at 37°C for 1 h. After infection, 2.5 mL of MEM(Selective) was added to each well and the plate returned to the 37°C incubator.

After 3-4 days of growth, the location of single, isolated plaques on the cell monolayer were marked on the underside of the tissue culture plate. The medium was removed, and the monolayer air-dried briefly. Using a 20 µL pipette tip containing 3 µL of MEM(F<sup>-</sup>A<sup>+</sup>), the plaque was gently scraped off the plate and transferred to a microfuge tube containing 50 µL of MEM(F<sup>-</sup>A<sup>+</sup>). The plaque preparation was freeze/thawed twice and stored at -80°C.

To amplify virus in isolated plaques, RK13 monolayers were prepared in a flat-bottomed 96-well plate. The monolayers were washed twice with MEM(F<sup>-</sup>A<sup>-</sup>). The plaque preparation was vortexed for 30 sec and added to the RK13 monolayers and incubated for 1 h at 37°C. The media was removed from the wells and 100 µL of MEM(Selective) added

to each well. The plate was incubated for 5-7 days, or until CPE was evident on the monolayer. The plate was then stored at -80°C.

#### *2.5.3.3 PCR screening of single isolated plaques*

To determine the purity of virus isolated from amplified plaques, PCR screening was used. A combination of two or three primers (Table 2.4) was used to identify recombinant and wild type (Uriarra) virus in each amplified virus preparation. The amplified virus in the 96-well plate was freeze/thawed twice, collected into a microfuge tube and vortexed for 30 sec. A 10 µL aliquot was transferred to a separate tube, microwaved for 2 min on high using a Sharp Carousel R-3C57 Microwave, cooled on ice and then vortexed for 30 sec. This plaque preparation (4 µL) was used as template in a 20 µL PCR as described in Section 2.1.8, and the PCRs separated by agarose gel electrophoresis as described in Section 2.1.5. Samples from which only PCR products indicative of recombinant virus, or predominantly PCR products from recombinant virus were amplified, were selected for further purification as described in Section 2.5.3.2. The process was repeated until only pure recombinant virus was present – a process that took between 3 and 7 rounds of plaque purification.

#### *2.5.3.4 Purification of recombinant virus using $\beta$ -glucuronidase (*gus*) selection*

Selection of recombinant virus using *gus* selection is reliant upon production of the *gus* gene product ( $\beta$ -glucuronidase) in cells and plaques formed from cells infected with recombinant virus staining blue in the presence of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-gluc). To isolate single recombinant plaques, the recombinant virus preparation was diluted and plated out on RK13 cell monolayers as described in Section 2.5.3.2, but without the use of selective reagents in the media (MEM[F<sup>-</sup>A<sup>+</sup>] only). After 4-5 days of growth, 10 µL of X-Gluc (10 mg/mL; Sigma, Australia) in 100 µL of MEM(F<sup>-</sup>A<sup>+</sup>)

**Table 2.4 – Primer combinations used in screening virus preparations for wild-type (Uriarra) and recombinant virus during purification.** A blank space indicates no product would be obtained for that virus type.

Virus	Primers	Wild Type Product Size (bp)	Recom. Product Size (bp)
Uriarra ΔM10L/M11L <i>gpt</i>	M009L-R1 x M012L-F1 x gpt-R1	1092	645
	M012L-F1 x M010L-R1	684	
	M012L-F1 x gpt-R1		645
Uriarra ΔMT-7 <i>gpt</i>	M007-R2 x M007-F1 x gpt-R1	747	603
	M007-F1 x M007-RI	380	
	M007-F1 x gpt-R1		603
Uriarra ΔMT-7 <i>gus</i>	M007-R2 x M007-F1 x gus-F1	747	411
	M007-F1 x M007-RI	380	
	M007-F1 x gpt-R1		411
Uriarra ΔM063R <i>gptgus</i>	M062R-F1 x M064R-R1 x gpt-R1	1223	440
	M064R-R1 x M063R-F1	660	
	M062R-F1 x gpt-R1		440

was added to each well. After 4-8 h further incubation, isolated blue plaques were marked, picked, amplified and screened by PCR as described previously for *gpt* selection.

#### **2.5.4 Preparation of concentrated virus**

Virus was grown in monolayers of RK13 or Vero cells in T180 cell culture flasks as described previously (Section 2.5.1) and collected into 40 mL Oak Ridge Tubes after freeze/thawing twice. The infected cell lysate was centrifuged for 2 h at 12,000 rpm in an SS-34 rotor (Sorvall RC5+ centrifuge) at 4°C. After centrifugation, the supernatant was poured off and the cell debris pellet resuspended in 1 mL of MEM(Complete). After vortexing for 60 sec, the concentrated virus preparation was sonicated (4 x 20 sec pulses, maximum power). The concentrated virus stock was then titred (Section 2.5.5) and stored at -80°C.

#### **2.5.5 Plaque assay to determine virus titre**

Virus stocks were titrated by plaque assay in duplicate on Vero cell monolayers and titres expressed as plaque forming units/mL (pfu/mL). Briefly, monolayers of Vero cells were prepared in 6-well plates and washed twice with 1 mL of MEM(F<sup>-</sup>A<sup>+</sup>). Ten-fold serial dilutions of the virus stocks were prepared, vortexing the virus for 20 sec between each dilution. Diluted virus (100 µL) was added to wells in duplicate, followed by 200 µL of MEM(F<sup>-</sup>A<sup>+</sup>). Plates were incubated for 1 hr at 37°C, rocking every 10 min, after which 2.5 mL of MEM(Complete) was added to each well. The plates were returned to the incubator at 37°C for 5-8 days. To visualise plaques, the media was poured off and replaced with 2 mL of 10% formalin in water and incubated at room temperature for 1 h. The formalin was then poured off and replaced with 1 mL of 0.1% crystal violet (Sigma, Australia) in water for 30 min. After staining, the fixed monolayers were washed liberally with water and left

to dry. Plaques could be observed, using a light box, as clear areas on a purple-stained cell monolayer. Plaques were counted and the concentration determined using the following equation:

Virus titre (pfu/mL) = average number of plaques per well x 10 x dilution factor

### **2.5.6 Virus growth curves in RK13 and Vero cells**

To measure the *in vitro* growth characteristics of recombinant viruses with respect to parental viruses, cells were infected *in vitro* and the titre of virus at specific times post-infection determined. Confluent monolayers of RK13 or Vero cells were prepared in 24-well plates ( $2 \times 10^5$  cells/well). Monolayers were washed twice with 1 mL of MEM(F<sup>-</sup>A<sup>+</sup>). Growth curves were performed at two MOIs: 0.1 and 3.0. To achieve this, concentrated virus stocks were diluted in MEM(F<sup>-</sup>A<sup>+</sup>) such that the concentration was  $2 \times 10^5$  pfu/mL (for MOI=0.1) and  $6 \times 10^6$  pfu/mL (for MOI=3), and 100 µL was plated onto duplicate wells. Seven identical plates were prepared simultaneously to obtain separate plates for each time point. Plates were incubated at 37°C for 1 h, rocking every 20 min, and then 400 µL of MEM(F<sup>-</sup>A<sup>+</sup>) added to each well. The final volume in each well was 500 µL. One plate was immediately stored at -80°C (Time 0 sample). The other plates were incubated at 37°C for 12, 24, 36, 48, 72 or 96 h, and one plate stored at -80 °C at each time point. The titre of virus in each sample was determined by plaque assay as described in Section 2.5.5.

### **2.5.7 Virus growth curves in RL-5 cells**

The *in vitro* growth characteristics of recombinant viruses with respect to parental viruses were also measured in the rabbit CD4<sup>+</sup> T lymphocyte cell line RL-5. RL-5 cells were infected *in vitro* and the titre of virus at specific times post-infection determined.

RL-5 cells were grown in RPMI medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 500 µg/mL amphotericin B, 60 µg/mL penicillin and 100 µg/mL streptomycin (RPMI[Complete]). When required for experiments, cells were transferred to a 50 mL Falcon tube and pelleted by centrifugation at 1500 rpm in a Beckman Allegra 6R centrifuge for 10 min. Cells were resuspended in RPMI(Complete) and counted using a haemocytometer. Cells were then diluted to a concentration of  $1 \times 10^6$  cells/mL. Viruses to be tested were diluted in RPMI(Complete) at a concentration of  $3 \times 10^6$  pfu/mL. Cells (5 mL) were mixed with virus (5 mL) in a 50 mL Falcon tube and incubated in a 37°C water bath for 60 min to infect (MOI = 3.0), gently mixing the tubes every 10 min. Following infection, duplicate 1 mL aliquots of each infection were placed in 24 well plates. One plate was immediately stored at -80°C (time 0 sample) and the others incubated at 37°C. Sample plates were frozen at 24, 48, 72 or 96 h post infection. The titre of virus in each sample was determined by plaque assay as described in Section 2.5.5.

### **2.5.8 Measuring the viability of RL-5 cells following infection with recombinant viruses**

Viability of infected RL-5 cells was measured by trypan blue exclusion assay. Viable cells do not take up trypan blue, but cells that have lost integrity (non-viable) stain blue. This method has been adapted from Macen *et al.* (1996). RL-5 cells were harvested and counted. Virus was added to  $5 \times 10^4$  cells such that the final MOI was 10 ( $5 \times 10^5$  pfu) in a final volume of 150 µL of RPMI(Complete). The mixture was incubated at 37°C for 1 h in a microfuge tube, inverting every 20 min, and then placed in a round-bottom 96-well plate and incubated at 37°C in a humidified, water-jacketed incubator with 5% CO<sub>2</sub>/95% air. A 25 µL aliquot was taken at 0, 3, 6, 12 or 24 h after infection and added to 25 µL of 0.4% trypan blue stain (GibcoBRL, USA). Using a haemocytometer, the total number of viable

(unstained) and non-viable (blue stained) cells was recorded and the percentage viability of cells calculated.

## **2.6 ANIMAL METHODS**

### **2.6.1 Rabbit care and housing**

Outbred domestic European rabbits (*Oryctolagus cuniculus*) used in this study were bred at the CSIRO Sustainable Ecosystems Animal House. Wild rabbits were also bred at the CSIRO Sustainable Ecosystems Animal House from populations captured from the wild near Canberra. They have been bred in captivity under specific pathogen free conditions for 2-4 generations. Animals were housed in an OGTR approved PC2 facility at a constant 20°C and 12 h light / 12 h dark regime. Rabbits were fed *ad libitum* on standard rabbit pellets supplemented with fresh cabbage and lettuce leaves.

### **2.6.2 Collecting blood samples from rabbits and processing**

Blood samples for serum (1 mL) were collected from the marginal ear vein using a 22G needle. The blood was stored at 4°C overnight then centrifuged at 13,000 rpm for 15 min and serum transferred to a fresh microfuge tube and stored at -20°C. Blood samples collected for isolation of peripheral blood mononuclear cells (PBMC; 10 mL) were collected from the marginal ear vein using a butterfly catheter (22G; Vacutainer, Australia) attached to a 10 mL syringe. Before blood collection, 1 mL of Heparin (1000 U/mL; Sigma, Australia) was drawn through the needle, catheter and syringe. Details of further processing are described in Section 2.7.1.



### **2.6.3 Vaccination, challenge and monitoring of rabbits**

Rabbits were inoculated with 50, 5000 or  $10^5$  pfu of vaccine virus in 100  $\mu$ L of PBS delivered intradermally at a site on the left thigh. Rectal temperatures were monitored daily, and the rabbits closely observed for any secondary signs of infection.

To determine the protective efficacy of vaccines in rabbits, animals were challenged with 1000 pfu of SLS intradermally at a site on the thigh. Rectal temperature was measured daily. The rabbits were closely observed for clinical signs of myxomatosis and scored using a scale of 0 to 5 (Kerr *et al.*, 2003). The designation for each number is described in Table 2.5. In every experiment, animals displaying severe symptoms (clinical score of 4.5 to 5) and not expected to survive were euthanased by intravenous injection of barbiturate.

### **2.6.4 Inoculation and tissue collection from rabbits for pathogenesis studies**

To determine the dissemination of virus *in vivo*, domestic or wild rabbits were inoculated intradermally with 5000 pfu of virus on the dorsal surface of the right hind foot. At 2, 4, 6, 10 or 20 days post inoculation, groups of three rabbits were euthanased by overdose of barbiturate delivered into the marginal ear vein. The following tissues were collected: skin from the inoculation site (primary lesion), the right popliteal lymph node (draining lymph node), skin from the left hind foot (distal skin), the left popliteal lymph node (contralateral lymph node) and left testis. Each tissue sample was divided in half: a sample was frozen immediately in preweighed tubes at  $-80^{\circ}\text{C}$  for virus titre determination (Section 2.6.6) and a sample was fixed in 10% formalin in PBS for histopathology (Section 2.6.5).

**Table 2.5 – Myxomatosis clinical scores and the corresponding symptoms.**

<b>Score</b>	<b>Symptoms</b>
<b>0</b>	No clinical signs of myxomatosis.
<b>0 to 0.5</b>	Reaction (reddening) at the inoculation site, through to a primary lesion less than 2 cm in diameter.
<b>0.5 to 1</b>	Primary lesion greater than 2 cm in size. No secondary signs of myxomatosis.
<b>1 to 2</b>	Very mild clinical signs limited to a slight reddening and swelling of eyelids.
<b>2 to 3</b>	Mild generalised signs of myxomatosis with swollen eyelids, some conjunctival discharge; mild anogenital swelling. Animals still bright and alert.
<b>3 to 4</b>	Moderate clinical signs of myxomatosis characterised by swollen eyelids with some mucopurulent discharge (eyes 0-75% closed); moderate anogenital swelling; secondary lesions on face and body. Animal appears depressed.
<b>4 to 5</b>	Severe myxomatosis. Eyes closed (75-100%) by swollen eyelids and copious mucopurulent discharge; nasal swelling; severe anogenital swelling; secondary lesions on face and body; very depressed demeanour; laboured, snuffly breathing.
<b>5</b>	Severely ill, not expected to survive.

### **2.6.5 Fixed tissue sections and histology**

The embedding, sectioning and haematoxylin and eosin staining of tissue sections were performed at the Histology Unit at the John Curtin School of Medical Research. Formalin-fixed tissues were embedded in paraffin wax. Two 5 µm sections were cut from each tissue and mounted on superfrost microscope slides (Menzel, Germany). Sections were dewaxed in three changes of xylene (BDH, Australia) for 2 min each and rehydrated through a graded series of ethanol (100% to 75%). Slides were washed in water and then stained with Gills' N° 3 haematoxylin (BDH, Australia) for 3 min. Slides were washed in water, stained with 0.025% alcoholic eosin for 2 min, differentiated in two washes of 90% ethanol and cleared in two washes of xylene. Sections were mounted under a coverslip with DPX (Difco, Australia), examined by light microscopy using an Olympus BX40 microscope and photographed using an Olympus DP11 colour digital camera.

### **2.6.6 Processing of tissue samples for determination of virus titre**

Tissue samples stored at -80°C (Section 2.6.4) were defrosted and weighed. Skin samples were minced with a scalpel blade and placed in 5 mL tubes with 4 volumes of 1 mg/mL collagenase D (Sigma, Australia) in PBS and incubated at 37°C for 2-3 h, vortexing every 30 min (Best and Kerr, 2000). After incubation, 5 volumes of MEM(Complete) were added to the samples. Other tissue samples were minced with a scalpel blade, placed in a microfuge tube, homogenised with a microfuge tube pestle (Bioventures, USA) and then mixed with 9 volumes of MEM(Complete). Both skin and other tissue samples were then sonicated using a Branson Sonifier 450 Cup sonicator (3 pulses of 15 sec, maximum power). Samples were then diluted and plaque assayed on Vero cell monolayers to determine the concentration of virus in samples, as described in Section 2.5.5, expressing the concentration of virus as plaque forming units per gram of tissue (pfu/g).

### **2.6.7 PCR analysis of virus in tissue biopsies**

Small samples of lesion material were collected using an 18G needle from both secondary and primary lesions. The material was placed in 200  $\mu$ L of PBS and subjected to three cycles of freeze/thawing, and then sonicated. Virus was amplified by placing 50  $\mu$ L of this material on RK13 cells in 6-well plates and incubating for several days under MEM(F<sup>-</sup>A<sup>+</sup>). After freeze/thawing several times, viral DNA was extracted using the Qiagen DNAeasy Tissue Kit as described in Section 2.5.2, and viral DNA analysed by PCR as described in Section 2.5.3.3.

## **2.7 MEASUREMENT OF IMMUNE RESPONSES IN VACCINATED RABBITS**

### **2.7.1 *In vitro* antigen-specific lymphocyte proliferation assay**

Peripheral blood lymphocytes (PBMC) were isolated from blood taken one week after the final boost of plasmid vaccine, and an antigen-specific proliferation assay performed as described in Han *et al.* (1999). Briefly, 10 mL of blood was collected from the marginal ear vein of rabbits into tubes containing 1000 U of heparin (Sigma, Australia) (Section 2.6.2), and then diluted with 10 mL of RPMI-1640 without any supplemental additives. This was gently layered over 10 mL of Lympholyte-Rabbit (Cedarlane, Canada) and centrifuged at 1000 g at room temperature for 30 min in a Beckman Allegra 6R centrifuge. PBMC were collected from the interface using a pasteur pipette, diluted to 20 mL with RPMI-1640 and centrifuged at 400 g for 15 min at 4°C to pellet cells. After removing the supernatant, contaminating red blood cells were lysed by the addition of 10 mL of ACK Shocking Buffer (155 mM NH<sub>4</sub>Cl [BDH, Australia], 10 mM KHCO<sub>3</sub> [BDH, Australia], 0.11 mM Na<sub>2</sub>EDTA, pH 7.3) and incubated on ice for 10 min. After shocking, 20 mL of RPMI(Complete) was added and the cells pelleted as previously described. PBMCs were washed with 20 mL of RPMI(Complete) three times and then counted using a

haemocytometer. They were resuspended in RPMI(Complete) and diluted to a final density of  $2 \times 10^6$  cells/mL.

Assays were performed in 96-well flat-bottomed microtiter plates (Nunc, USA). For each PBMC sample, at least four sets of wells were set up in quadruplicate. Into each group of four wells was placed 100  $\mu$ L of cells ( $2 \times 10^5$  cells) and one of the following solutions: concanavalin A (10  $\mu$ L of a 5  $\mu$ g/mL solution in PBS; Sigma, Australia) as a positive control for lymphocyte proliferation and viability; 10  $\mu$ L of PBS (negative control); 10  $\mu$ L of purified myxoma virus antigen (25  $\mu$ g/mL in PBS; Section 2.3); 10  $\mu$ L of negative control antigen (25  $\mu$ g/mL in PBS; Section 2.3). Cells were incubated at 37°C in a humidified, water-jacketed incubator in 5% CO<sub>2</sub>/95% air for 5-6 days. Twenty-four hours prior to harvest, cells were pulsed with 1.0  $\mu$ Ci of <sup>3</sup>H-thymidine in 20  $\mu$ L of RPMI(Complete). Cells were harvested onto glass filters and <sup>3</sup>H-thymidine incorporation measured as described in Section 2.4.8. The antigen-specific stimulation index was calculated for each animal using the formula below:

$$\text{Stimulation index (SI)} = \frac{[\text{Average cpm in wells in the presence of recombinant antigen}]}{[\text{Average cpm in wells in the presence of negative control antigen}]}$$

The statistical significance of each stimulation index value was determined using Fieller's theorem to determine the upper and lower limits of the 95% confidence interval (Finney, 1971). For the purposes of this study, an SI value for which the lower limit of the 95% confidence interval was greater than 1.5 was considered a positive antigen-specific response.

### **2.7.2 Detection of antigen-specific antibody responses in DNA-vaccinated animals**

Antibody responses to M055R and M121R in DNA-vaccinated rabbits were measured using immunofluorescent methods as described in Section 2.4.5, except 1:50 dilutions of vaccinated rabbit serum were used on pDual-55R- or pDual-121R-transfected RK13 cells respectively. FITC-conjugated anti-rabbit Ig (Silenus Labs, Australia) was used as secondary antibody at a concentration of 1:100. Binding of rabbit antibody to transfected cells was observed under a Leitz Fluovert FS microscope. The observation of increased specific fluorescence compared to pre-vaccination serum from the same rabbit was recorded as a positive seroconversion.

Antibody responses to M055R, M073R and M115L in vaccinated rabbits were detected using immunoblotting. Purified recombinant antigen (production described in Section 2.3) was run on a 12% SDS-PAGE gel, transferred onto a PVDF membrane and probed with 1:200 dilutions of vaccinated rabbit serum, using the protocol described in Section 2.2. Binding of antibody was detected using HRP-conjugated anti-rabbit Ig (Silenus Labs, Australia), and ECL+ Chemiluminescence kit (Amersham, UK). An increase in binding of antibody to the recombinant protein band compared to pre-vaccination serum from the same rabbit was recorded as a positive seroconversion.

### **2.7.3 Enzyme-linked immunosorbent assay (ELISA)**

To determine the anti-myxoma virus antibody levels in vaccine virus-infected rabbits, whole virus ELISA was used as described in Kerr (1997). A laboratory stock of myxoma virus (strain Lausanne) was purified by ultracentrifugation. Lausanne was grown in RK13 cells in 180 cm<sup>2</sup> tissue culture flasks and concentrated as described in Section 2.5.4, except the pellet was resuspended in PBS before sonication. The virus preparation was digested

with DNase (25 µg/mL; Sigma, Australia) and RNase (50 µg/mL; Sigma, Australia) for 30 min at 37°C with frequent agitation. Virus was then pelleted through a step-gradient of 0.54 mL of dextran solution (10% dextran T10 [Amersham Pharmacia Biotech, Sweden], 10 mM Tris.HCl, 1 mM EDTA, pH 8.0) over which was layered 0.56 mL of sucrose solution (36% sucrose (BDH, Australia), 10 mM Tris.HCl, 1 mM EDTA, pH 8.0). The virus preparation was layered over the top of this gradient and centrifuged at 250,000 g for 20 min in a Beckman TLS-55 rotor. The pellet was resuspended in PBS and the step-gradient centrifugation repeated. The final pellet was resuspended in 1 mL of PBS and stored at -20°C.

This stock was diluted 1:12,500 with carbonate buffer (15 mM Na<sub>2</sub>CO<sub>3</sub> [BDH, Australia], 35 mM NaHCO<sub>3</sub> [BDH, Australia], pH not adjusted). This dilution was found to give a final OD<sub>405</sub> reading of 1.0 using a 1:100 dilution of standard serum from myxoma virus-infected rabbits. This pooled serum was used within the lab to calibrate new stocks of myxoma virus antigen. Aliquots of diluted virus (50 µL) were added to each well of an Immulon 2HB ELISA plate (Immulon, USA) and incubated at 4°C overnight. The wells were then washed three times with PBS. The wells were then blocked with 100 µL of ELISA blocking buffer (PBS containing 5% skim milk powder and 0.05% Tween 20) for 2 h at 37°C. The plate was then washed four times with Tween-PBS (PBS containing 0.05% Tween 20).

Serum samples from rabbits were serially diluted from 1/100 to 1/10,2400 with ELISA antibody binding buffer (PBS containing 0.05% Tween 20 and 1% skim milk powder). Duplicate aliquots of 50 µL were added to the prepared Immulon plates and incubated at 37°C for 2 h. Wells were then washed six times with Tween-PBS. HRP-conjugated anti-

rabbit Ig (Silenus Labs, Australia) was used as a secondary antibody. This was diluted 1/100 with ELISA antibody binding buffer and 50  $\mu$ L aliquots added to each well and incubated at 37°C for 30 min. The plate was then washed six times with Tween-PBS and tapped dry on paper towelling. The HRP substrate was prepared by dissolving a 10 mg 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) [ABTS; Sigma, Australia] tablet in 10 mL of citrate-phosphate buffer (61.4 mM citric acid [BDH, Australia], 77 mM Na<sub>2</sub>HPO<sub>4</sub> [Ajax, Australia], pH 4.0) and adding 20  $\mu$ L of 30% hydrogen peroxide solution [BDH, Australia]. The substrate solution (100  $\mu$ L) was added to each well and incubated at room temperature for 10 min. The absorbance at 405 nm was then measured using a  $\mu$ Quant Plate Spectrophotometer, with OD<sub>405</sub> values zeroed against a 1/100 dilution of pooled sera from uninfected rabbits. The reciprocal of the highest serum dilution giving an average absorbance above 0.1 was recorded as the anti-myxoma virus antibody titre. If the average absorbance at the lowest dilution (1/100) was less than 0.1, the rabbit was recorded as having an anti-myxoma virus antibody titre of zero.

#### **2.7.4 Plaque reduction neutralisation assay (PRNA)**

The titre of myxoma virus-neutralising antibody was measured by a plaque reduction neutralisation assay as described in Kerr (1997). Serum from rabbits was heated to 56°C for 10 min to inactivate complement prior to testing. Serum was then serially diluted from 1/5 to 1/10240 with MEM(F<sup>-</sup>A<sup>-</sup>) and 150  $\mu$ L aliquots of each dilution added to microfuge tubes. A previously titred stock of Lausanne was diluted in MEM(F<sup>-</sup>A<sup>-</sup>) to a final concentration of 2000 pfu/mL, and 150  $\mu$ L aliquots of this diluted virus stock added to each diluted serum sample. This resulted in final serum dilutions of 1/10 to 1/20480. A negative control serum was prepared by diluting pooled and heat-treated serum from uninfected rabbits with MEM(F<sup>-</sup>A<sup>-</sup>) such that the final concentrations were 1/10 and 1/100.



A third, serum-free negative control was also prepared. The tubes were incubated at 37°C for 1 h, vortexing briefly every 20 min. During this time, 6-well plates with confluent monolayers of Vero cells were prepared by washing monolayers once with MEM(F<sup>-</sup>A<sup>-</sup>). After the incubation, 100 µL of the virus/serum dilutions were added to wells in duplicate, and incubated at 37°C for 1 h, rocking the plate every 10 min. The virus/serum dilution was then aspirated, replaced with 2.5 mL of MEM(Complete) and incubated for 8 days at 37°C in a 5% CO<sub>2</sub>/95% air humidified water-jacketed incubator. Visualisation of plaques was achieved as described in Section 2.5.5.

The percentage virus neutralisation at each serum dilution was calculated as follows:

$$\% \text{ neut.} = 100 - \left[ \frac{\text{Mean number of plaques on plates with test serum}}{\text{mean number of plaques per plate on negative control plates}} \times 100 \right]$$

The 50% and 80% neutralisation titres for each serum sample were determined as the reciprocal of the highest serum dilution at which greater than 50% or 80% of plaques were neutralised, respectively.

## **Chapter 3**

# **Construction and testing of DNA vaccines expressing selected myxoma virus antigens**

## CHAPTER 3 – CONSTRUCTION AND TESTING OF DNA VACCINES

### EXPRESSING SELECTED MYXOMA VIRUS ANTIGENS

The material presented in this chapter has been accepted for publication:

Adams, M.M., van Leeuwen, B.H. and Kerr, P.J. (2004). "Limitations of plasmid vaccines to complex viruses: selected myxoma virus antigens as DNA vaccines were not protective." Vaccine: In press. Available online at: <http://dx.doi.org/10.1016/j.vaccine.2004.05.023>

#### 3.1 INTRODUCTION

The endogenous production of antigen by DNA vaccine-transfected cells can result in a strong Th1-type immune response and can induce high levels of antigen-specific CTLs, with demonstrated protective efficacy in many disease models (Shedlock and Weiner, 2000). As cell-mediated responses are thought to be critical in protecting rabbits against myxomatosis, the ability of DNA vaccines expressing myxoma virus antigens to protect rabbits from myxomatosis was investigated. The effect on vaccine efficacy of the co-expression of the rabbit cytokines interleukin-2 (IL-2) and interleukin-4 (IL-4) with virus antigens was also investigated. In previous studies in other animal models, IL-2 cDNA co-delivery has enhanced both cell-mediated and antibody responses to DNA immunogens and resulted in a skewing towards Th1-like responses (Pan *et al.*, 1999). Similarly, IL-4 expressing DNA vaccines have previously been shown to increase the seroconversion rate and improve antibody titres to DNA immunogens, and bias the immune response toward Th2 development (Pan *et al.*, 1999). As well as a potential direct path to vaccine development, this study also sheds light on the possible protective antigens of poxviruses and expands studies into a different model from the VV/mouse model where antigen-specific studies have been performed to date.

This chapter describes the selection of myxoma virus antigens for testing as DNA vaccines, construction of the vaccines, *in vitro* testing of viral antigen expression, purification of the DNA vaccine plasmids and the purification of viral antigen for use in the *in vitro* analysis of antigen-specific immune responses in rabbits. The vaccines were then administered to domestic European rabbits (*Oryctolagus cuniculus*). Following vaccination, the antigen-specific antibody and T cell immune responses to individual myxoma virus antigens were determined, and the rabbits then challenged with a virulent strain of myxoma virus to assess the protective efficacy of the various antigen/cytokine combinations.

## 3.2 RESULTS

### 3.2.1 Antigen Selection

The large size of the poxvirus genome, the complexity of its structure and the unknown functions of many genes means that little work has been done to identify protective antigens of poxviruses until recently. Protective antigens have been identified from research undertaken using the model Orthopoxvirus, vaccinia virus (VV) (Demkowicz *et al.*, 1992; Galmiche *et al.*, 1999; Hooper *et al.*, 2000; Hooper *et al.*, 2003; Lai *et al.*, 1991b; Pulford *et al.*, 2004). Most of these antigens are membrane proteins of the intracellular mature virion (IMV) membrane or the extracellular enveloped virion (EEV) membrane. The IMV membrane in VV possesses 13 integral or associated proteins – A9L, A28L, A13L, A14L, A14.5, A17L, A27L, D8L, D13L, E10R, H3L, I5L and L1R (Moss, 2002). The EEV membrane in VV possesses five unique proteins – A33R, A34R, F13L, A56R and B5R (Moss, 2002; Smith *et al.*, 2002). Homologues of all these antigens except D8L are found in myxoma virus (Cameron *et al.*, 1999).

Eleven antigens have been shown to protect, or partially protect, mice from lethal VV challenge (L1R, D8L, H3L, A33R, A27L, A4L, A10L, A56R, A36R and B5R) (Demkowicz *et al.*, 1992; Galmiche *et al.*, 1999; Hooper *et al.*, 2000; Hooper *et al.*, 2003; Lai *et al.*, 1991b; Pulford *et al.*, 2004) or are targets of neutralising antibodies (A27L, D8L, L1R, H3L, A17L, B5R) (Galmiche *et al.*, 1999; Gordon *et al.*, 1991; Hsiao *et al.*, 1999; Ichihashi *et al.*, 1994; Rodriguez and Esteban, 1987; Wallengren *et al.*, 2001; Wolffe *et al.*, 1995). These antigens were initially identified by the induction of high titres of antibody against these antigens during natural infection (Demkowicz *et al.*, 1992), or by the ability of monoclonal antibodies directed against these antigens to neutralise virus *in vitro* (Lai *et al.*, 1991b) or protect mice from challenge when passively administered (Hooper *et al.*, 2000).

Five myxoma virus antigens homologous to the VV antigens L1R, H5R, A17L, A27L and A33R were selected for initial screening and three more were placed on a secondary list (H3L, A10L and B5R). These antigens (M055R, M073R, M107L, M115L, M121R, M071L, M099L and M144R respectively), their known or putative functions, and the results of vaccination experiments are summarised in Table 3.1.

L1R (M055R), A27L (M115L) and A33R (M121R) have been demonstrated to protect mice from lethal challenge with VV when delivered as DNA vaccines (Galmiche *et al.*, 1999; Hooper *et al.*, 2000; Hooper *et al.*, 2003; Pulford *et al.*, 2004). A27L and A33R have also been shown to confer complete protection from lethal VV challenge when delivered as protein vaccines (Demkowicz *et al.*, 1992; Galmiche *et al.*, 1999; Lai *et al.*, 1991b). These results suggest that these three antigens may be excellent vaccine candidates. A17L (M107L) has not been studied as a vaccine, but monoclonal antibodies directed against the

**Table 3.1** – Putative functions, location and protective efficacy of candidate myxoma virus antigens. Antigens were selected based on homology to VV antigens that were shown to protect mice from lethal VV challenge or were targets of neutralising antibody.

Myxoma Virus Gene	VV Gene	Identity / similarity of proteins (%) <sup>a</sup>	Location in Virus	Function	Vaccination or protection experiments	References
<b>M055R</b>	L1R	68.2 / 82.6	IMV	IMV attachment and penetration	Neutralising antibody; partially protective DNA vaccine	Wolffe <i>et al.</i> (1995) Ichihashi and Oie (1996) Hooper <i>et al.</i> (2000) Hooper <i>et al.</i> (2003) Pulford <i>et al.</i> (2004)
<b>M073R</b>	H5R	42.4 / 59.2	Viriosome-associated, cytoplasmic	Late <i>trans</i> -activator factor (VLTf-4)	Not done – originally misidentified as target of potent neutralising antibody. The true target is H3L.	Beaud <i>et al.</i> (1995) Beaud and Beaud (1997) Kovacs and Moss (1996) Chertov <i>et al.</i> (1991)
<b>M107L</b>	A17L	43.3 / 62.6	IMV	Virion morphogenesis	Neutralising antibody	Rodriguez <i>et al.</i> (1993) Rodriguez <i>et al.</i> (1997) Wallengren <i>et al.</i> (2001)
<b>M115L</b>	A27L	26.7 / 57.1	IMV	Virus-cell fusion; EEV morphogenesis	<i>In vivo</i> neutralising antibody; <i>E. coli</i> -produced protein vaccine protective; DNA vaccine partially protective	Rodriguez and Esteban (1987) Rameriz <i>et al.</i> (2002) Lai <i>et al.</i> (1991b) Demkowicz <i>et al.</i> (1992) Hooper <i>et al.</i> (2003) Pulford <i>et al.</i> (2004)
<b>M121R</b>	A33R	36.1 / 58.4	EEV	Cytoplasmic motility via actin tails; direct cell-cell spread	Protective DNA vaccine	Roper <i>et al.</i> (1998) Galmiche <i>et al.</i> (1999) Hooper <i>et al.</i> (2000) Pulford <i>et al.</i> (2004)
<b>Secondary candidates</b>						
<b>M071L</b>	H3L	34.9 / 61.0	IMV	Immunodominant IMV antigen	Neutralising antibody; <i>E. coli</i> -produced protein vaccine of the capripox homologue (p32) partially protected goats from capripox challenge	Gordon <i>et al.</i> (1988) Gordon <i>et al.</i> (1991) Chertov <i>et al.</i> (1991) Carn <i>et al.</i> (1994) Pulford <i>et al.</i> (2004)
<b>M099L</b>	A10L	49.9 / 69.0	Virion Core	Component of virion core	Protein vaccine protected 75% of mice from lethal VV challenge	Heljasvaara <i>et al.</i> (2001) Demkowicz <i>et al.</i> (1992)
<b>M144R</b>	B5R	26.6 / 46.4	EEV	Actin tail formation, cell-cell spread	DNA vaccine protective; neutralising antibody protective <i>in vivo</i>	Mathew <i>et al.</i> (1998) Engelstad <i>et al.</i> (1992) Galmiche <i>et al.</i> (1999) Hooper <i>et al.</i> (2003) Pulford <i>et al.</i> (2004)

a - Identity and similarity values were calculated using Bestfit.

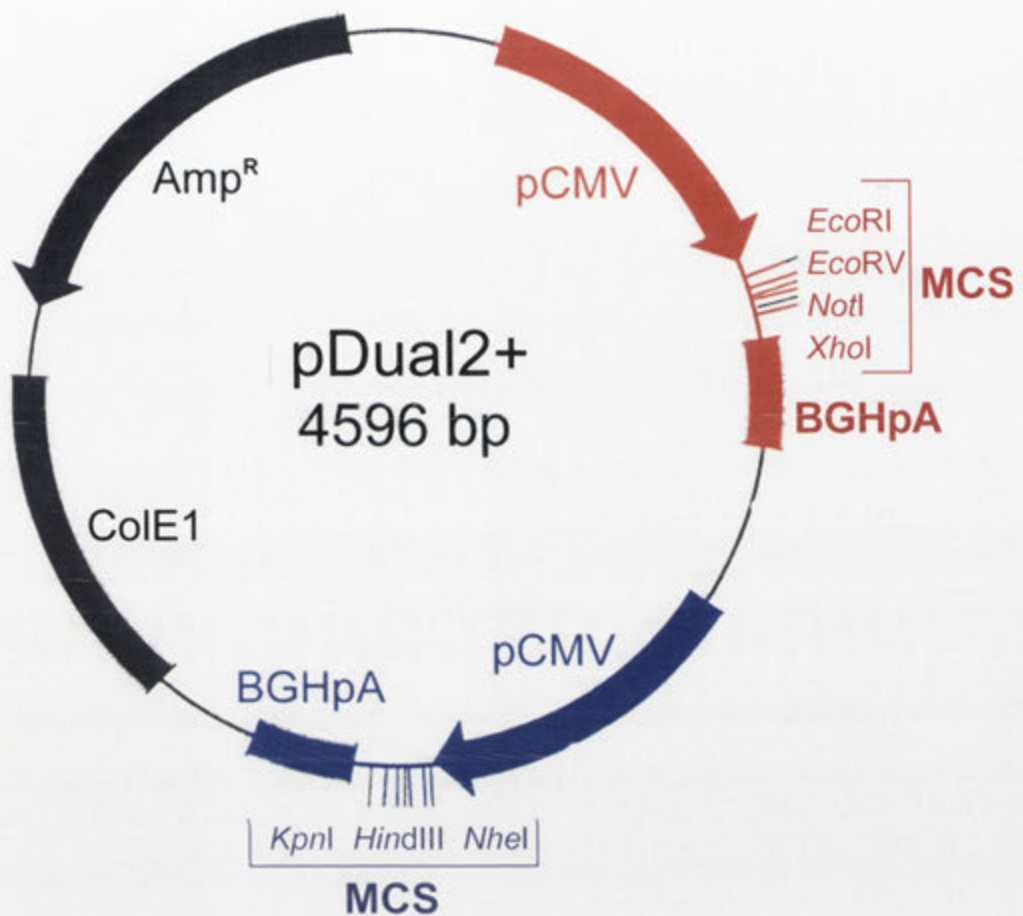
N-terminal region of A17L were able to neutralise infection of VV (Wallengren *et al.*, 2001), suggesting an important role for this antigen in VV pathogenesis. For this reason, M107L was selected as a vaccine candidate.

The myxoma virus homologue of H5R (M073R) was selected based on research which suggested H5R was a highly immunogenic IMV membrane antigen and the target of potent neutralising antibodies (Gordon *et al.*, 1988; Gordon *et al.*, 1991). However, after the vaccination trials described in this thesis had commenced, further analysis of the literature revealed that H5R had been misidentified, the immunogenic antigen being the co-migrating 35 kDa antigen, H3L (M071L) (Chertov *et al.*, 1991; Zinoviev *et al.*, 1994). It is interesting to note that the misidentification of H5R has been perpetuated in several later peer-reviewed papers and reviews (Hooper *et al.*, 2000; Moss, 1996). As M073R vaccination trials had already commenced, they were completed as planned.

### **3.2.2 Cloning of myxoma virus genes, and construction of candidate vaccine plasmids**

The vector selected for use as the DNA vaccine backbone was pDual2+ (obtained from Dr Scott Thomson, John Curtin School of Medical Research, Australian National University). This vector contains two expression cassettes derived from pcDNA3.1+, each controlled by a cytomegalovirus immediate-early promoter (CMV-IE) followed by a bovine growth hormone polyadenylation signal (BGHpA), between which is located a multiple cloning site (Figure 3.1). The two expression cassettes allow for the constitutive co-expression of two different genes from the same vaccine construct. In this study, myxoma virus genes were cloned into the 5' expression cassette and cytokine genes into the other.





**Figure 3.1 - The DNA vaccine vector pDual2+.** This 4596 bp vector has two separate expression cassettes each containing the cytomegalovirus intermediate-early promoter (pCMV), a multiple cloning site (MCS) and bovine growth hormone polyadenylation signal (BGHpA). Each MCS encodes sites for different restriction enzymes as shown. Other sequences include the ampicillin resistance gene (Amp<sup>R</sup>) and the colicin E1 replicon (ColE1). Myxoma virus antigen genes were cloned into the first (red), and rabbit cytokine cDNAs into the second (blue) multiple cloning sites.



Myxoma virus DNA was prepared from the Lausanne strain of the virus, whose genome has been completely sequenced (Cameron *et al.*, 1999). Primers were designed to amplify the ORFs of the five selected genes, and to add an *EcoRI* site immediately before the start codon, and a *XhoI* site a few bases after the stop codon (Table 2.2). *Pfu* polymerase was used to amplify the antigen genes, which were then were digested with *EcoRI* and *XhoI* and cloned into the first expression cassette of pDual2+. The constructs were sequenced, confirming that the nucleotide sequence of the antigen genes was identical to the reported Lausanne sequence (Cameron *et al.*, 1999) and that the orientation of the antigen genes was correct with respect to the promoter sequence (data not shown).

Rabbit IL-2 and IL-4 cDNAs were individually amplified from plasmids (supplied by Dr Harvey Perkins, School of Biochemistry and Molecular Biology, Australian National University). Primers were designed with a *NheI* site immediately before the start codon and a *HindIII* site immediately after the stop codon (Table 2.2). After amplification, the cDNAs were digested with *NheI* and *HindIII* and ligated into pDual2+. The correct orientation and nucleotide sequence of the cytokine cDNA in each plasmid was confirmed by sequencing (data not shown).

Vaccine plasmids were constructed for each of the five antigens containing IL-2, IL-4 or no cytokine. Plasmids containing IL-2 or IL-4 with no antigen gene were also prepared. Therefore, with the vector pDual2+, 18 vaccine plasmids were prepared. Each vaccine plasmid was named by the antigen and cytokine expressed; for example pDual-55R-IL2 expresses both M055R and rabbit IL-2.

A control plasmid expressing influenza haemagglutinin (HA) derived from the influenza strain A/Puerto Rico/8 was also constructed. The gene was amplified from a plasmid (supplied by Dr Sue McKenzie, CSIRO Sustainable Ecosystems) and cloned into the first expression cassette of pDual2+. This plasmid was called pDual-HA.

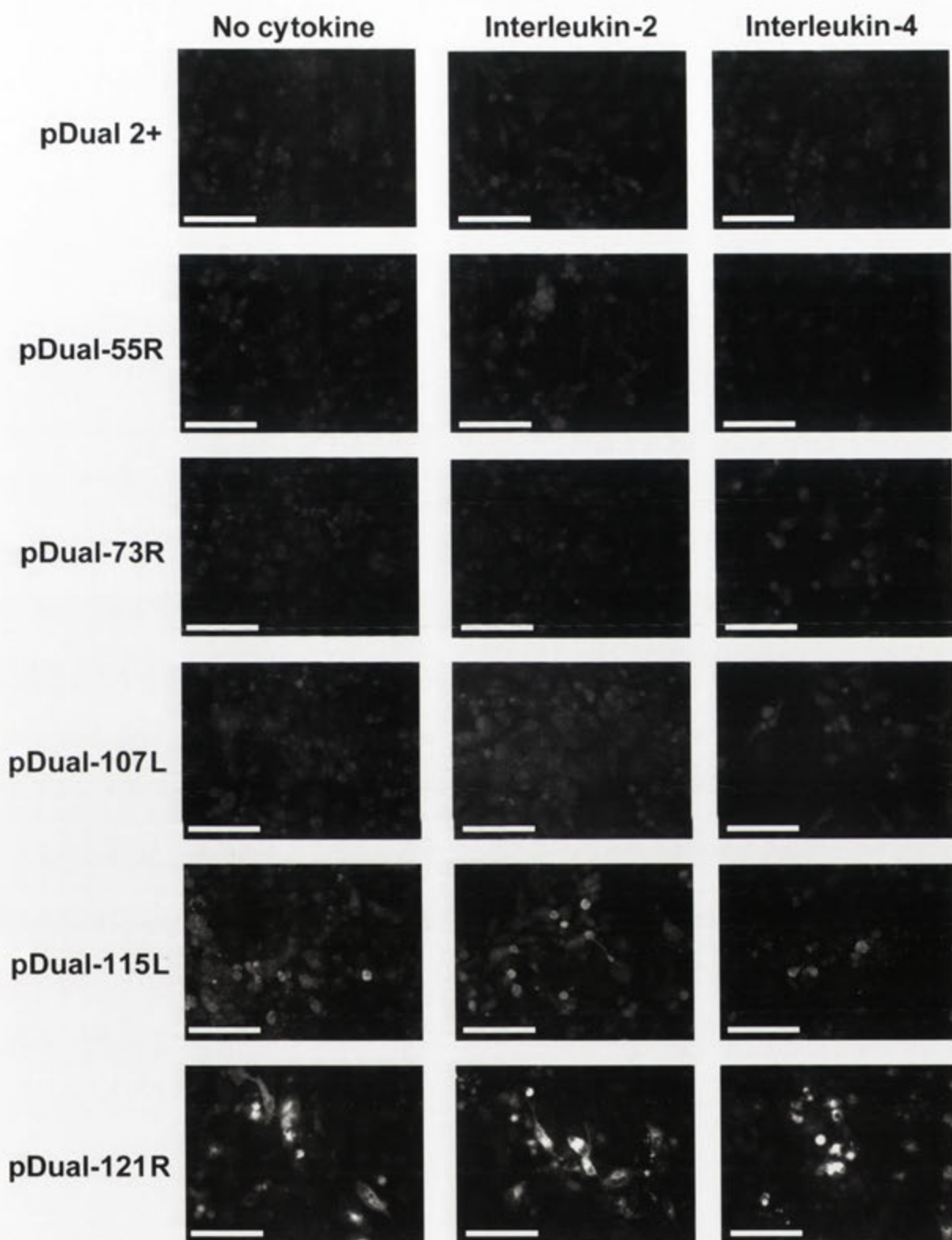
### **3.2.3 Confirmation of antigen production in vaccine-transfected cells**

To confirm that the vaccine constructs were producing each myxoma virus antigen, RK13 cells were transfected with each of the vaccine plasmids. Vaccine plasmid DNA was prepared as described in Section 2.1.3.1 and transfections performed as described in Section 2.4.2. These transfected cells were used in a number of techniques to detect the presence of antigen protein and antigen gene transcripts.

#### *3.2.3.1 Immunofluorescent detection of antigens*

Twenty-four hours after transfection, RK13 cells transfected with the plasmid vaccines were fixed with methanol:acetone, and probed with a 1:400 dilution of polyclonal anti-myxoma virus serum (Section 2.4.5). This serum contains antibodies to at least 20 myxoma virus antigens, detectable on an immunoblot (data not shown).

Figure 3.2 shows RK13 cells transfected with each of the 18 vaccine plasmids and probed with the polyclonal anti-myxoma virus serum. There was no specific fluorescence observed in cells transfected with plasmids expressing M055R, M073R or M107L (Figure 3.2). M115L expression did not result in as intensely fluorescent individual cells, but there is an increase in fluorescent intensity when compared to pDual2+-transfected cells (Figure 3.2, fifth row). M121R expression resulted in intensely fluorescent, readily visible transfected cells (Figure 3.2, bottom row). There was a high level of background



**Figure 3.2 - Detection of plasmid vaccine-expressed myxoma virus antigens using cellular immunofluorescence.** Cells transfected with plasmid vaccines were probed with polyclonal anti-myxoma virus serum (1:400 dilution) and anti-rabbit FITC-conjugated secondary antibody. Results are shown for each of the 18 vaccine plasmids constructed, containing either no antigen gene (top row) or the myxoma virus antigen genes M055R (second row), M073R (third row), M107L (fourth row), M115L (fifth row) or M121R (bottom row), and co-expressing no cytokine (first column), rabbit IL-2 (middle column) or rabbit IL-4 (last column). Bars on images are 200  $\mu$ m in length.

fluorescence even on control cells (pDual2+-transfected) which may have masked low-level specific fluorescence due to the expression of the first three antigens. Alternatively, the polyclonal anti-myxoma virus serum used may only contain very low levels of antibodies specific for M055R, M073R and M107L.

Expression of HA from RK13 cells transfected with pDual-HA was confirmed by cellular immunofluorescence using 1:100 diluted serum from a rabbit vaccinated with VV expressing the same HA gene (VV-HA; Kerr and Jackson, 1995) (Figure 3.3). Cells expressing HA were observed as brightly fluorescing bodies in the cell monolayer. No immunofluorescence was observed on cells transfected with pDual2+.

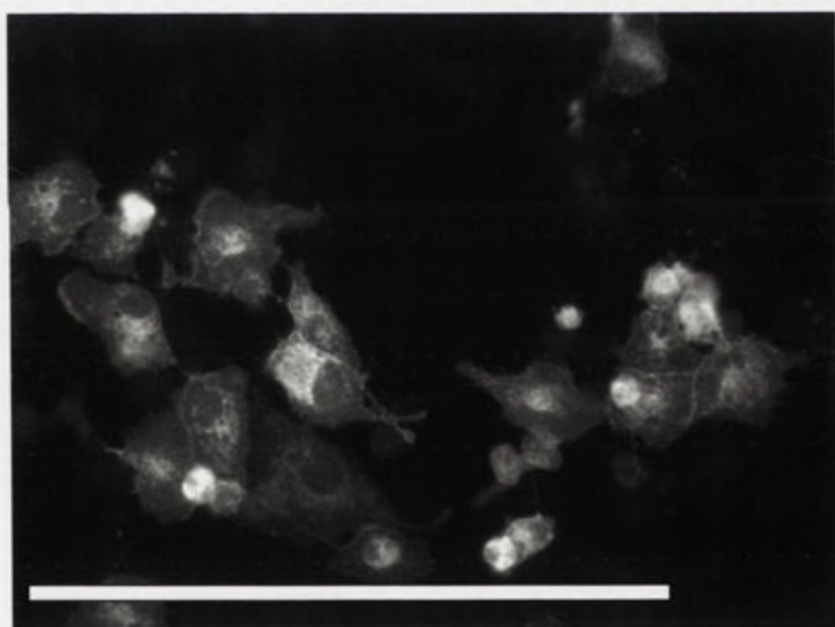
#### *3.2.3.2 Immunoblot detection of myxoma virus antigens*

Immunoblotting is an alternative method of detecting expression of proteins. Twenty-four hours after transfection, total cellular protein was collected from vaccine-transfected RK13 cells by solubilisation in SDS-PAGE sample loading buffer under reducing (0.1M DTT) or non-reducing conditions. After separation on a SDS-PAGE gel and blotting, the membrane was probed with the polyclonal anti-myxoma virus serum (1:1000 dilution). Specific binding of antibody was observed in the lysates of cells transfected with pDual-M115L under both reducing and non-reducing conditions and expression of M073R was visible under reducing conditions (Figure 3.4), although the intensity was not as pronounced. This may be due to lower anti-M073R antibody titres in the polyclonal serum, or differing expression levels of the two antigens. The sizes observed for both M073R and M115L (37 and 34 kDa respectively), were larger than those expected based on protein sequence (21.5 and 21.6 kDa respectively) although this does not take into account post-translational

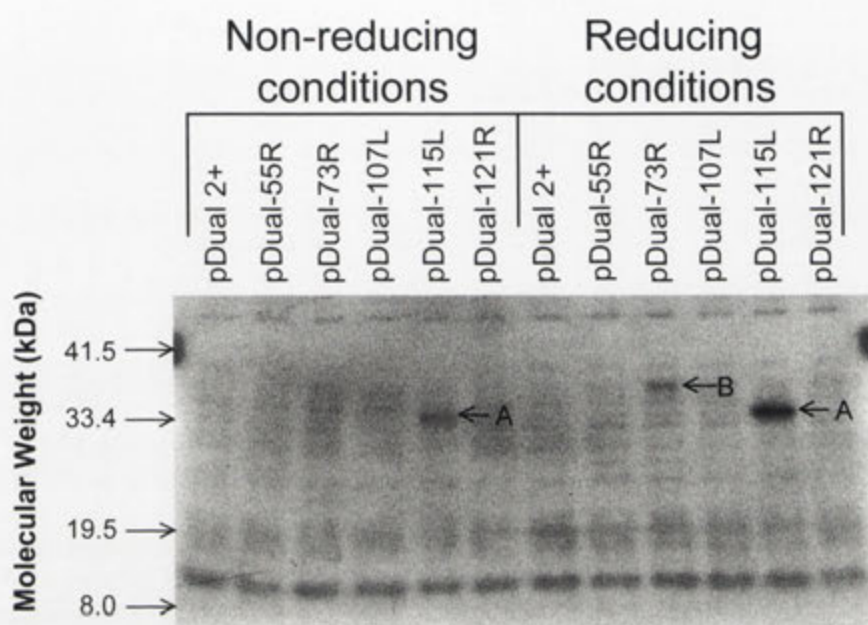
**A**



**B**



**Figure 3.3 - Confirmation of expression of influenza HA in RK13 cells transfected with pDual-HA.** RK13 cells were transfected with pDual2+ (A) or pDual-HA (B) and probed with serum (1:100 dilution) collected from a rabbit infected with VV expressing the same influenza HA gene (VV-HA). Bars on images are 200  $\mu\text{m}$  in length.



**Figure 3.4 -** Detection of plasmid vaccine-expressed myxoma virus antigens by immunoblot. Cells transfected with each of the myxoma virus antigen-expressing plasmids (without cytokine co-expression) were grown for 24 hours, total cellular protein was extracted under non-reducing or reducing (0.1M DTT) conditions, separated on an SDS-PAGE gel, blotted onto a PVDF membrane and probed with polyclonal anti-myxoma virus serum (1:1000 dilution). The expression of M115L (A) and M073R (B) are arrowed.

modification. Similar increases in molecular weight were observed for the VV homologues, H5R (22.3 to 35 kDa) and A27L (12.6 to 14 kDa) (Johnson *et al.*, 1993).

Specific binding of antibody to the lysates of cells transfected with pDual-55R, pDual-107L and pDual-121R was not observed on immunoblots. Anti-M121R antibodies in the polyclonal serum may not be able to bind to the protein under denaturing conditions, although strong specific binding was evident on methanol-acetone fixed cells (Figure 3.2). M055R and M107L expression was not detected using either antibody-mediated detection method. It was possible that the proteins were not detectable due to very low or absent titres of anti-M055R and anti-M107L antibodies in the polyclonal serum. However, another possibility is that the antigen genes are not being transcribed appropriately.

#### 3.2.3.3 Northern analysis to detect myxoma virus antigen transcripts

Poxviruses replicate in the cytoplasm of infected cells. A large part of the poxvirus genome codes for proteins required for its own replication and transcription (Moss, 2002). For this reason, the mRNA transcribed from the poxvirus genome is never exposed to the splicing machinery of the nucleus. However, in the context of a DNA vaccine, the antigen genes are transcribed in the nucleus under the control of the CMV-IE promoter, and therefore genes could potentially be inappropriately spliced. It was possible that the M055R and M107L genes contained splice-consensus sequences, which would result in no mature mRNA and therefore no protein.

The five myxoma virus candidate genes were analysed by the program GeneSplicer ([http://www.tigr.org/tdb/GeneSplicer/gene\\_spl.html](http://www.tigr.org/tdb/GeneSplicer/gene_spl.html)) which screens DNA sequences for the presence of potential splice-consensus sequences (Pertea *et al.*, 2001). The entire myxoma

virus antigen sequences cloned into pDual2+, including restriction enzyme sites and 3' untranslated sequence was analysed by this program using splice-consensus sequences for humans, as there is no data available on rabbit splice-consensus sequences (Table 3.2). Although the software found several low probability potential splice sites in each gene, no antigen gene seemed more likely than any other to contain splice-consensus sequences. The highest probability donor sites, as defined by the highest scores, were found in M115L and acceptor sites in M107L (Table 3.2). These sites were only defined as Medium confidence. Therefore, sequence analysis did not strongly suggest the presence of splice-consensus sequences in M055R and M107L.

Northern analysis was used to directly detect antigen transcripts in DNA vaccine-transfected cells as described in Sections 2.1.7. RK13 cells were transfected with each of the vaccine plasmids and incubated for 24 hours. Total RNA was extracted, separated on an agarose/formaldehyde gel, blotted onto a nylon membrane and probed with DIG-labelled antigen DNA.

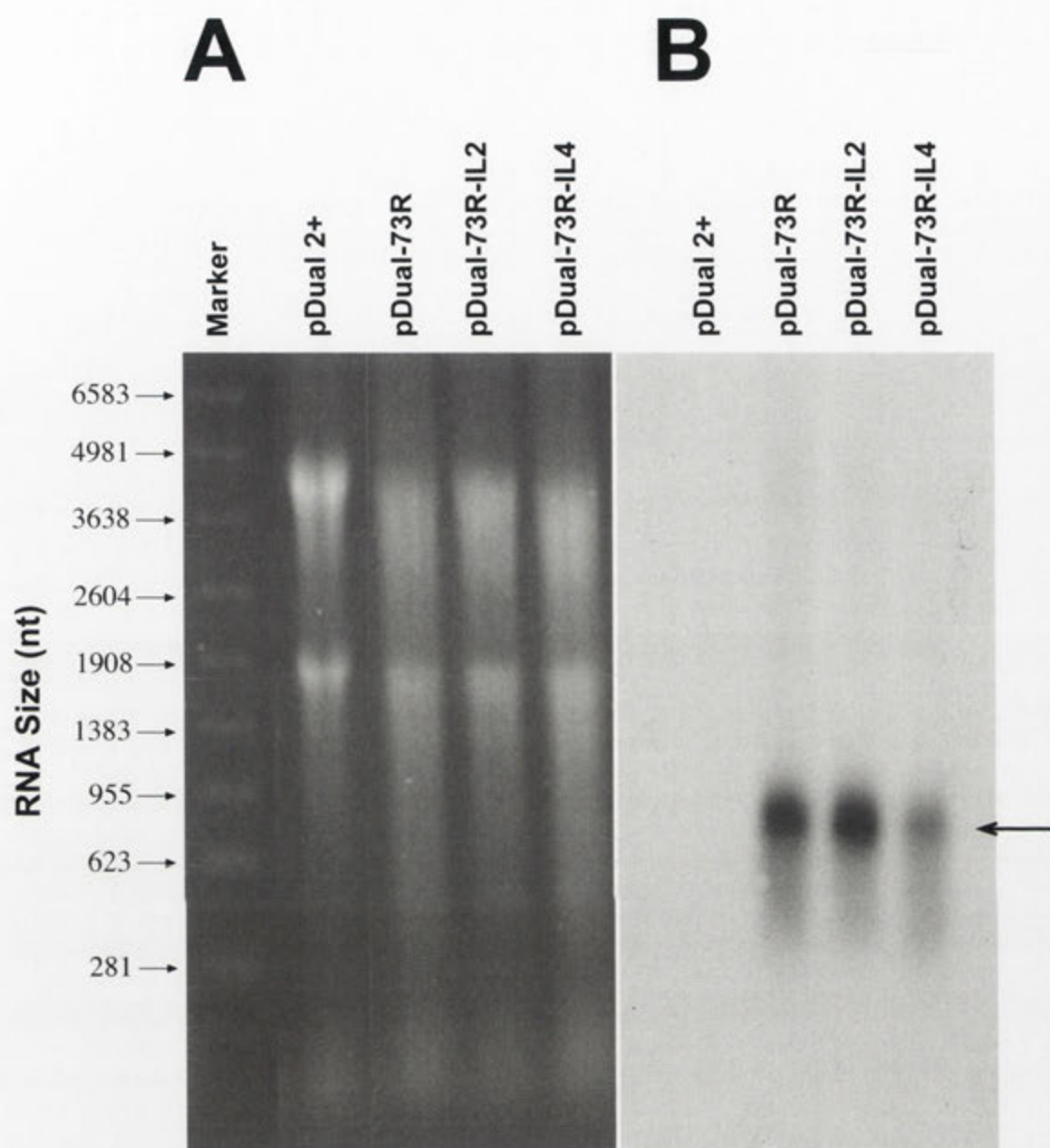
The sizes of the open reading frames of M073R and M121R are 622 and 567 bp respectively. After probing RNA extracted from cells transfected with M073R-expressing plasmids with randomly primed DIG-labelled M073R DNA, a distinct band of approximately 850 nt was detected (Figure 3.5). RNA extracted from cells transfected with M121R-expressing vaccines was probed with randomly primed, DIG-labelled M121R DNA, and an approximately 800 nt product was detected (Figure 3.6). These results indicate that the mRNA transcripts are approximately 230 nt longer than the open reading frame of the cloned gene, the extra length originating from 5' and 3' untranslated DNA generated by the CMV-IE promoter and BGH polyadenylation signal. A slightly larger



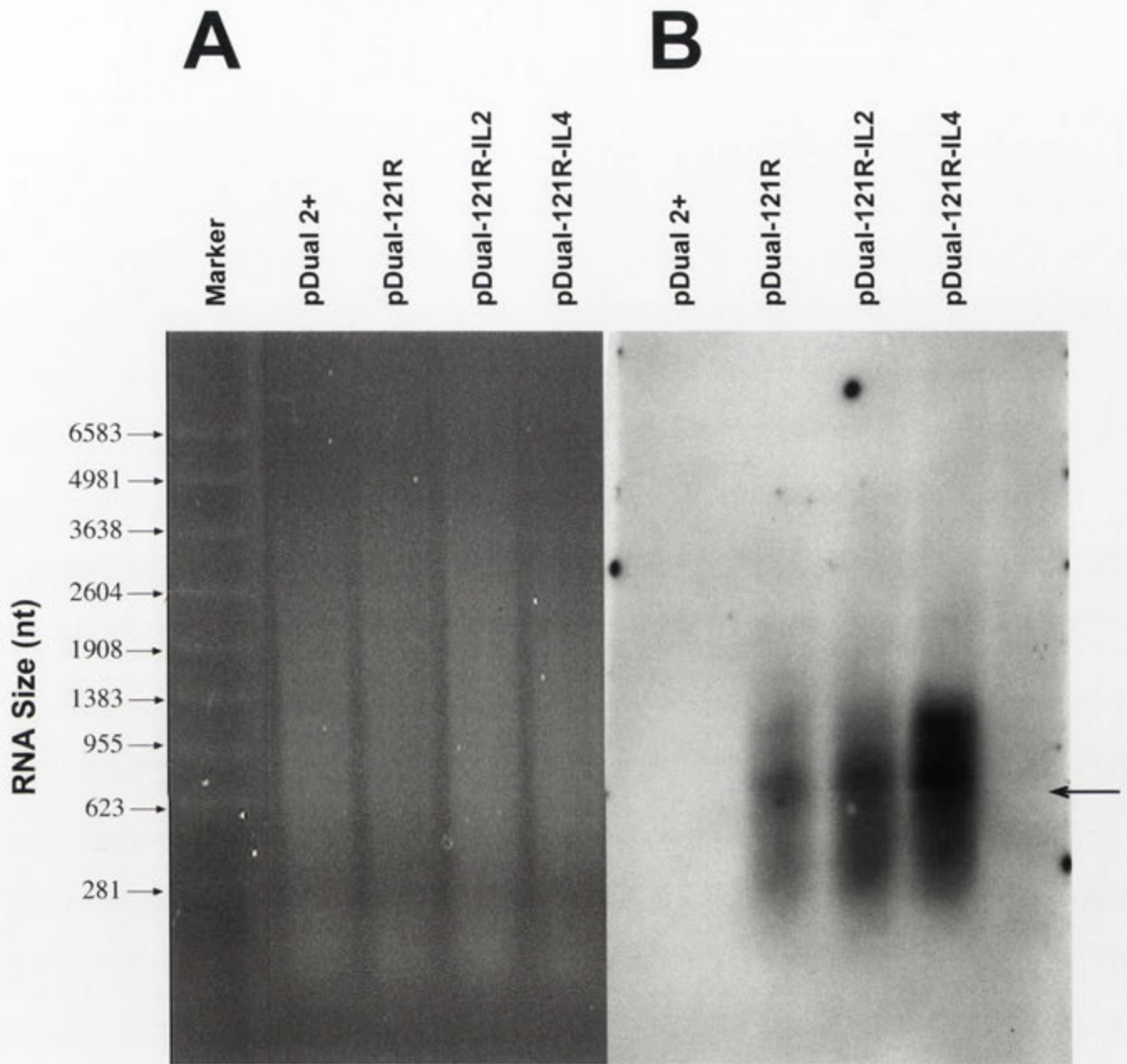
**Table 3.2 - Potential donor and acceptor splice sites in cloned myxoma virus genes as determined by GeneSplicer.**

Gene	Donor Sites		Acceptor Sites	
	Position <sup>a</sup>	Score <sup>c</sup> / Confidence <sup>d</sup>	Position <sup>a</sup>	Score <sup>c</sup> / Confidence <sup>e</sup>
<b>M055R</b> 747 bp <sup>b</sup>	261	3.86 / Medium	106	4.37 / Medium
<b>M073R</b> 600 bp <sup>b</sup>	182	3.88 / Medium	198 504	5.44 / Medium 5.13 / Medium
<b>M107L</b> 622 bp <sup>b</sup>			132 356 480	4.67 / Medium 10.20 / Medium 2.97 / Medium
<b>M115L</b> 592 bp <sup>b</sup>	223	4.86 / Medium	506	4.11 / Medium
<b>M121R</b> 556 bp <sup>b</sup>	275 431	2.32 / Medium 3.80 / Medium	89 433	2.14 / Medium 3.27 / Medium

- a – Position refers to the nucleotide number within the submitted sequence including the *EcoRI* site immediately before the start codon (start of gene = position 7).
- b – Length of sequence submitted to GeneSplicer including restriction sites and 3' untranslated sequence cloned into pDual2+.
- c – The score as defined by GeneSplicer is a measure of the probability of the site being a true splice consensus sequence.
- d - A high confidence donor site has a score of greater than 14.16. Scores greater than zero are of medium confidence.
- e - A high confidence acceptor site has a score of greater than 13.22. Scores greater than zero are of medium confidence.



**Figure 3.5 - Northern analysis of M073R mRNA expression in RK13 cells transfected with vaccine plasmids.** Total RNA from RK13 cells transfected with vaccines containing the M073R gene were separated on a formaldehyde-agarose gel, blotted onto a nylon membrane and probed with randomly primed, DIG-labelled M073R DNA. **A:** Ethidium bromide stained agarose gel showing loading of RNA isolated from cells transfected with vaccine plasmids. **B:** After transfer to a PVDF membrane and probing with DIG-labelled M073R DNA, an approximately 850 nt product was detected on the autoradiograph (arrowed).

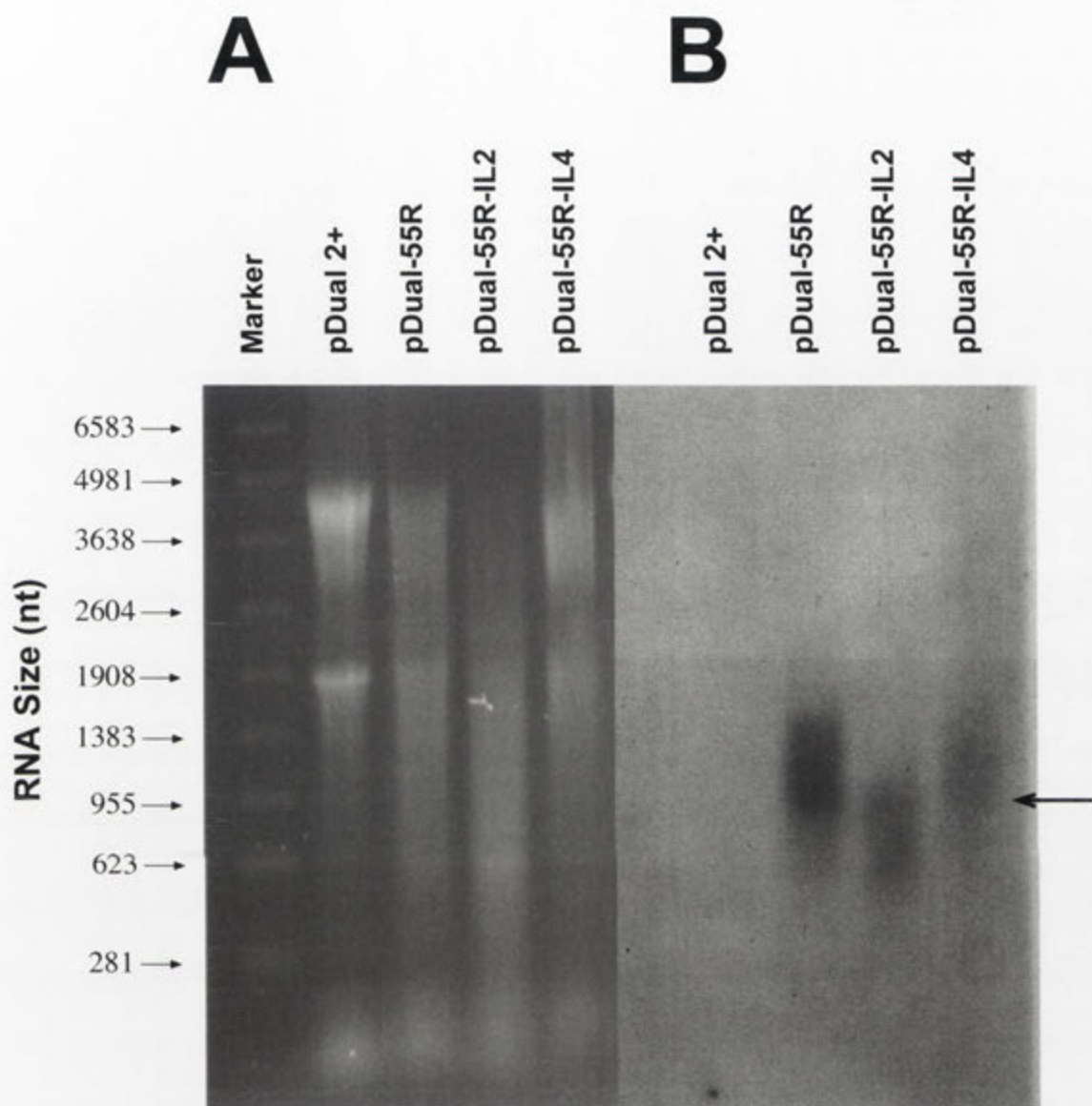


**Figure 3.6 -** Northern analysis of M121R mRNA expression in RK13 cells transfected with vaccine plasmids. Total RNA from RK13 cells transfected with vaccines containing the M121R gene were separated on a formaldehyde-agarose gel, blotted onto a nylon membrane and probed with randomly primed, DIG-labelled M121R DNA. **A:** Ethidium bromide stained agarose gel showing loading of RNA isolated from cells transfected with vaccine plasmids. **B:** After transfer to a PVDF membrane and probing with DIG-labelled M121R DNA, an approximately 800 nt product was detected on the autoradiograph (arrowed).

band of approximately 1000 nt was also observed in RNA isolated from pDual-121R-IL4 transfected cells; the identity of this band is not known.

The size of the mRNA transcript from cells transfected with vaccines expressing M055R (768 bp ORF) was expected to be approximately 1000 nt, which included 5' and 3' untranslated sequences. When RNA from cells transfected with M055R-expressing plasmids was probed with DIG-labelled M055R DNA, an approximately 1000 nt product was detected (Figure 3.7). This suggested that these cells were producing full length mRNA coding for M055R. The band from pDual-55R transfected cells was distinct, but in other lanes was not as clear, and did not reproduce well in the autoradiograph in Figure 3.7B. The intensity of the bands appeared to be lower than for other the other two myxoma virus genes – approximately 20% of the intensity when compared to M073R exposed for the same amount of time (data not shown).

No M107L RNA was detected using Northern analysis, although several attempts were made (data not shown). The failure to detect M107L protein or mRNA transcripts suggested that this antigen was not being produced in cells transfected with M107L-expressing plasmid vaccines. Therefore, M107L-expressing vaccines were not analysed further or used in animal vaccination experiments. Northern detection of M115L transcripts was not performed as protein expression had been demonstrated by immunofluorescence and immunoblotting.



**Figure 3.7 - Northern analysis of M055R mRNA expression in RK13 cells transfected with vaccine plasmids.** Total RNA from RK13 cells transfected with vaccines containing the M055R gene were separated on a formaldehyde-agarose gel, blotted onto a nylon membrane and probed with randomly primed, DIG-labelled M055R DNA. **A:** Ethidium bromide stained agarose gel showing loading of RNA isolated from cells transfected with vaccine plasmids. **B:** After transfer to a PVDF membrane and probing with DIG-labelled M055R DNA, an approximately 1000 nt product was detected on the autoradiograph (arrowed).

### **3.2.4 Confirmation of cytokine production from vaccine-transfected cells**

To confirm the expression of rabbit IL-2 and IL-4 from cells transfected with vaccine plasmids, RK13 cells were transfected with vaccine plasmids as described in Section 2.4.2 and examined for the presence of the cytokines.

As no antibodies were available to detect rabbit IL-2, a bioassay able to detect rabbit IL-2 was used (Section 2.4.8). The mouse T cell line HT-2 has an absolute dependence on IL-2 for growth (Wadhwa *et al.*, 1995). Rabbit IL-2 has been shown to induce the proliferation of this cell line (Dr Peter Kerr, personal communication). Supernatants from RK13 cells transfected with IL-2-containing plasmids were able to induce high levels of proliferation in HT-2 cells (Figure 3.8), and levels were equivalent between vaccines co-expressing the various myxoma virus antigens. This method was not quantitative for rabbit IL-2, and not indicative of how much rabbit IL-2 was produced.

Expression of rabbit interleukin 4 (IL-4) was confirmed using a polyclonal anti-peptide antibody directed against Helix-A of rabbit IL-4. This anti-rabbit IL-4 antibody is able to detect rabbit IL-4 in both cellular immunofluorescence and immunoblotting (Kerr *et al.*, 2004). As shown in Figure 3.9, there were high levels of specific fluorescence on cell monolayers transfected with IL-4-expressing vaccines, indicating the expression of rabbit IL-4.

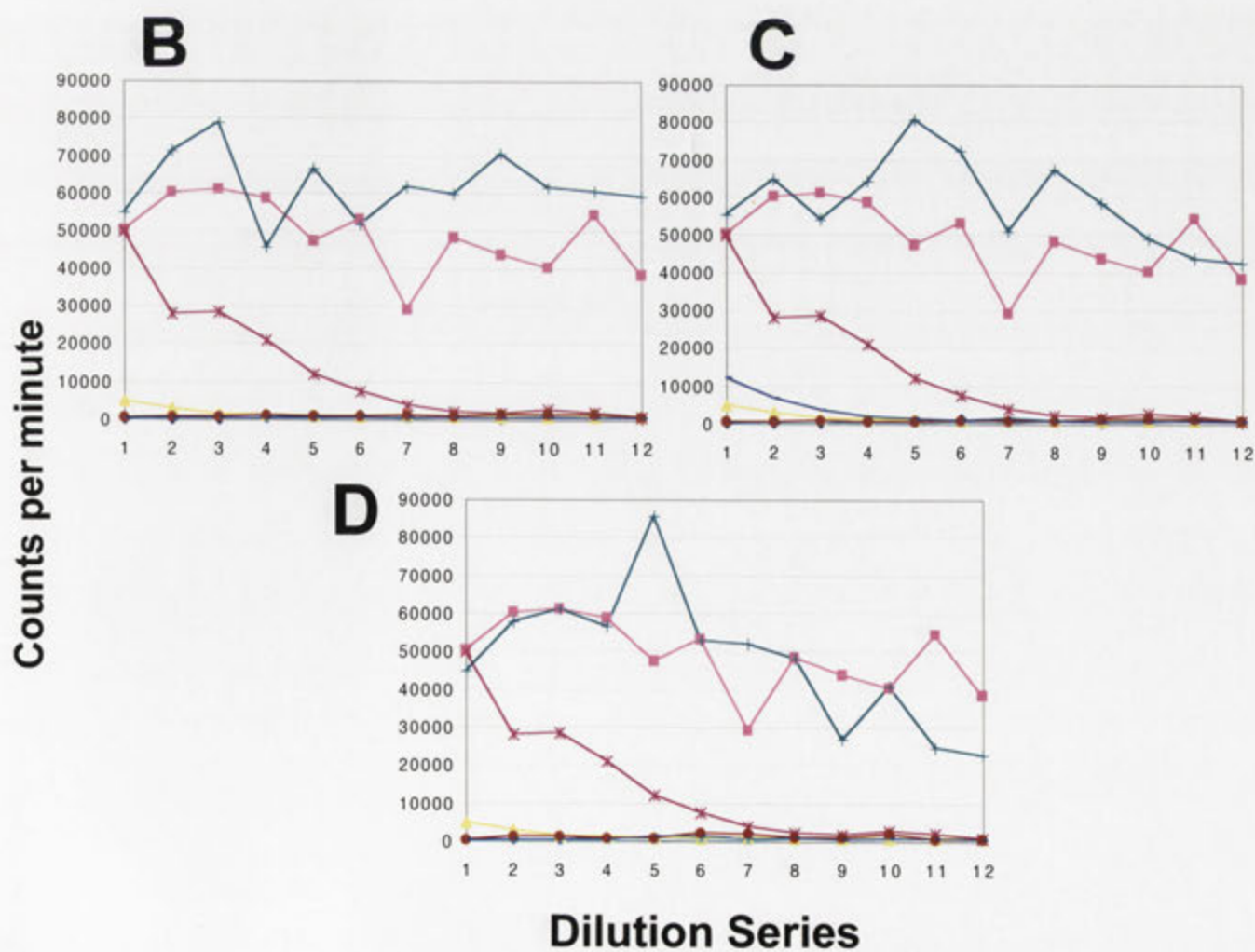
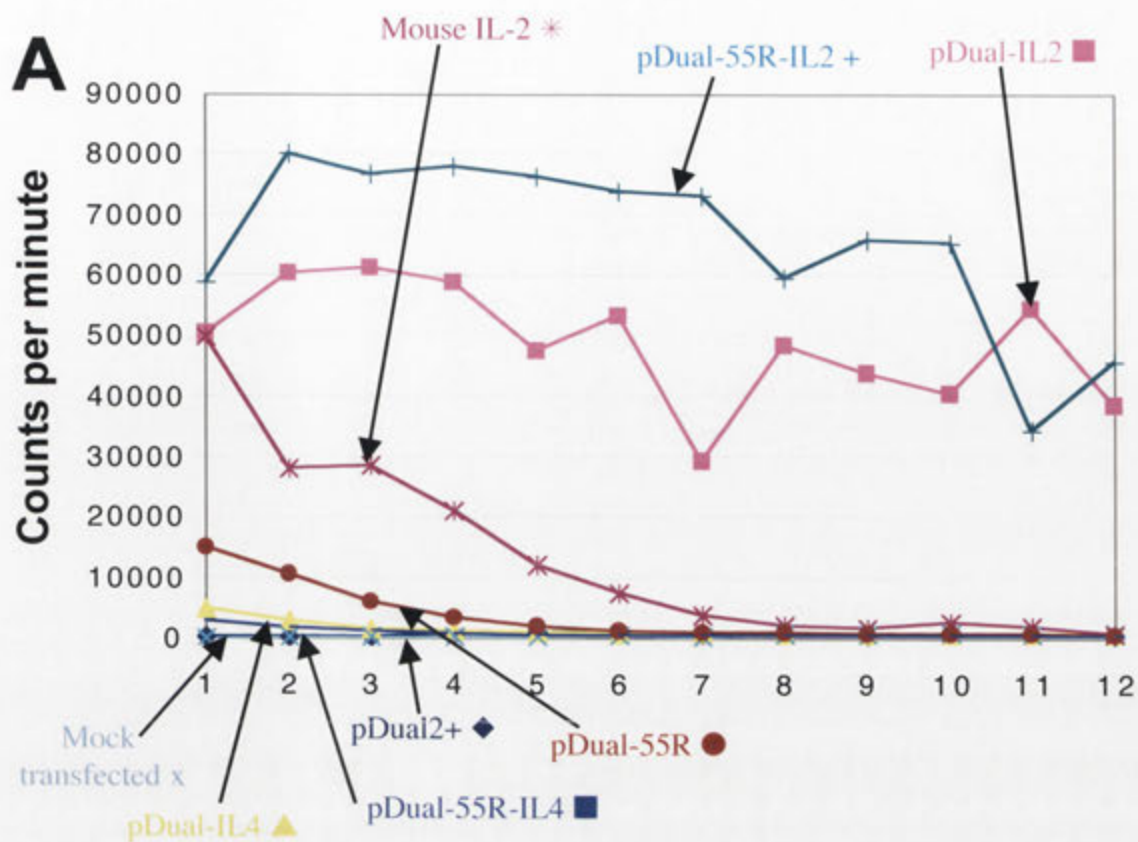
### **3.2.5 Purification of vaccine plasmids for injection**

Each vaccine plasmid was purified by two rounds of CsCl gradient ultracentrifugation (Section 2.1.3.2). Generally, 7.5-10 mg of plasmid was obtained from 4 L of *E. coli* culture. For injection, plasmids were resuspended in normal saline, and the concentration

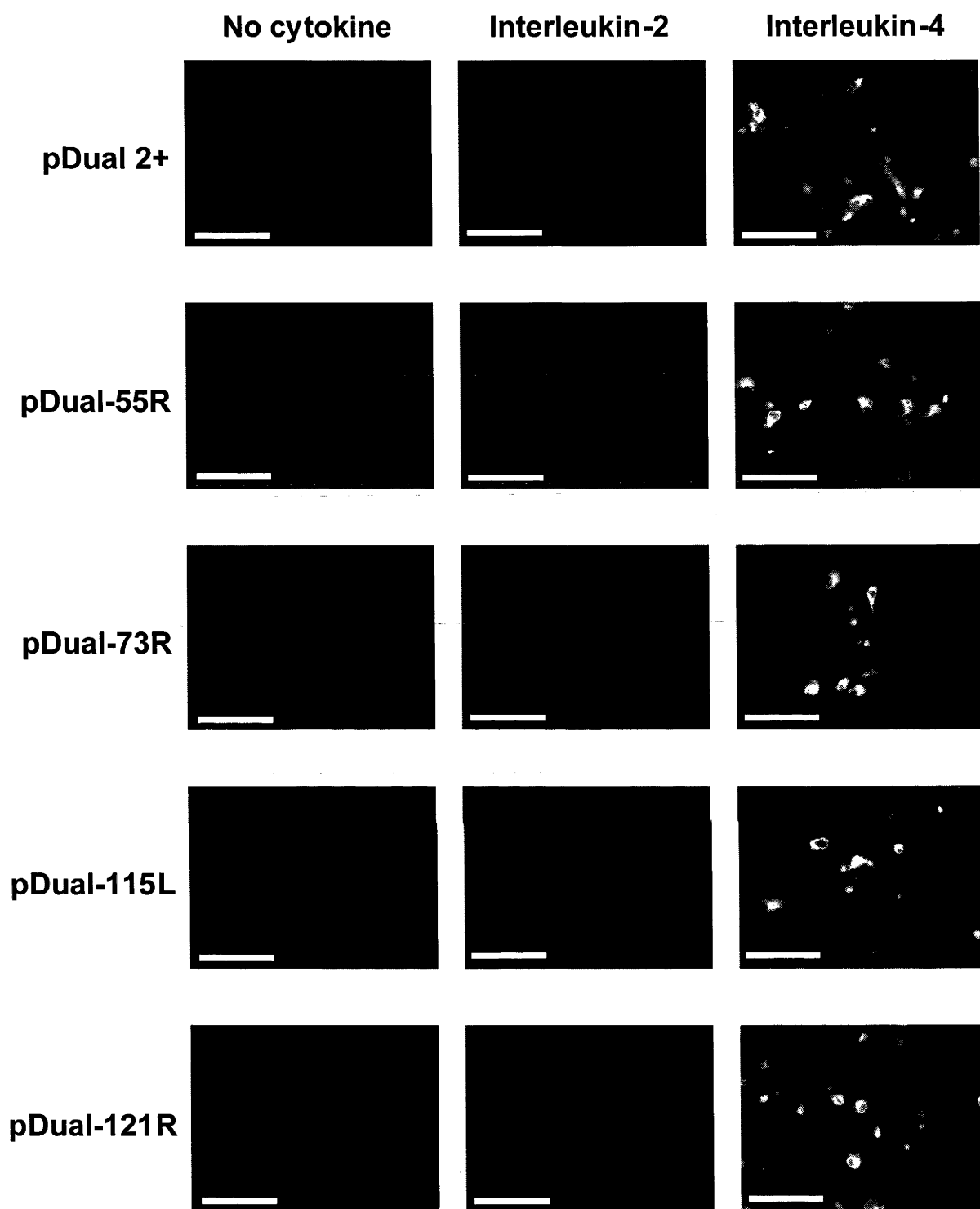
**Figure 3.8 - HT-2 bioassay for IL-2 production from plasmid vaccine-transfected cells.** DNA vaccines were transfected into RK13 cells and incubated for 48 hours. Supernatants were collected, concentrated, and applied to the IL-2-dependent cell line HT-2 in a two-fold dilution series. The degree of HT-2 proliferation was measured using  $^3\text{H}$ -thymidine incorporation, as indicated by measured counts per minute. DNA vaccines expressing M055R (A), M073R (B), M115L (C) and M121R (D) are shown with the corresponding controls. Graph A has been enlarged to show each tested supernatant in detail. There was little  $^3\text{H}$ -thymidine incorporation by cells treated with supernatants from mock-transfected, pDual2+-transfected, pDual-IL4-transfected, pDual-Antigen-transfected and pDual-Antigen-IL-4-transfected cells (A-D).

Key: mock transfected - ✕; pDual 2+ - ◆; mouse IL-2 - \*; pDual-IL2 - ■; pDual-IL4 - △; pDual-Antigen - ●; pDual-Antigen-IL2 - +; pDual-Antigen-IL4 - ■.









**Figure 3.9 - Detection of rabbit IL-4 expression in cells transfected with plasmid vaccines using cellular immunofluorescence.** IL-4 expression was detected using a polyclonal anti-rabbit IL-4 serum (1:100 dilution) and a FITC-conjugated secondary antibody. Results are shown for each of the 18 vaccine plasmids constructed containing no antigen gene (top row) or the myxoma virus antigen gene M055R (second row), M073R (third row), M115L (fourth row) or M121R (bottom row), and co-expressing no cytokine (first column), rabbit IL-2 (middle column) or rabbit IL-4 (last column). Bars on images are 200  $\mu$ m in length.

adjusted to 1 mg/mL. The purity of the plasmid was confirmed by restriction enzyme digestion and agarose gel electrophoresis (data not shown) and absence of endotoxin by Pyrotell Gel-Clot assay (Associates of Cape Cod, USA).

### 3.2.6 Vaccination of rabbits

Each vaccine was administered to groups of three male, outbred domestic rabbits. The vaccine plasmid (1 mg/mL in saline) was injected intramuscularly (250 µL) into each thigh (a total dose of 500 µg of plasmid DNA).

In a previous study, mice vaccinated with DNA vaccines expressing both an IMV and an EEV antigen were more effectively protected from lethal challenge with VV than by vaccination with either antigen alone (Hooper *et al.*, 2000; Hooper *et al.*, 2003). To determine if vaccination with more than one myxoma virus antigen was able to elicit a greater protective immune response than a single antigen, groups of three rabbits were vaccinated with both M115L (an IMV antigen) and M121R (an EEV antigen). The vaccination regime was the same as for other vaccine groups, except 500 µg of *each* plasmid was injected at each boost. M115L and M121R-expressing plasmids co-expressing rabbit cytokines were also co-delivered.

To confirm that the vaccination method was able to elicit antigen-specific immune responses in rabbits, the plasmid expressing influenza haemagglutinin (pDual-HA) was administered to rabbits as described for the other DNA vaccines. This antigen is highly immunogenic and has elicited potent antibody and cell-mediated immune responses in a range of animal species when delivered as a DNA vaccine (Bot *et al.*, 1999; Chen *et al.*, 1999; Lunn *et al.*, 1999; Operschall *et al.*, 1999; Radu *et al.*, 1999).

These doses were repeated at 2, 4 and 6 weeks after the initial inoculation. No reaction was observed at the inoculation site after the first or subsequent doses of vaccine plasmid.

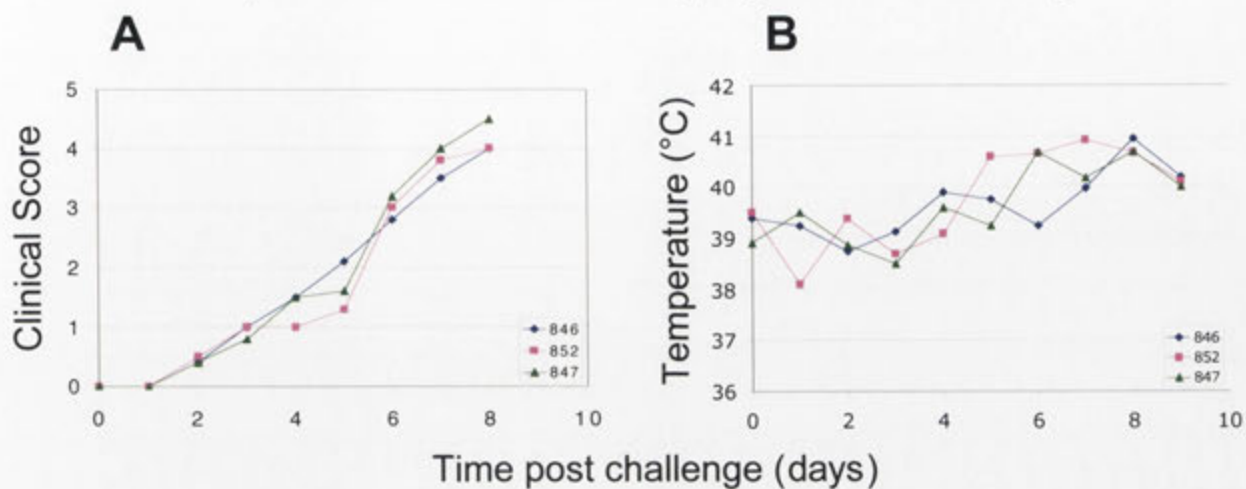
### **3.2.7 Challenge of rabbits with Standard Laboratory Strain (SLS) of myxoma virus**

All DNA-vaccinated rabbits, except those vaccinated with pDual-HA, were challenged by subcutaneous injection of 1000 pfu of SLS on the thigh and monitored for clinical signs of myxomatosis for up to 13 days post infection (dpi). This dose is 100% lethal for domestic rabbits within 10 to 13 days (Robinson *et al.*, 1999). A clinical score (CS) was recorded as a measure of disease progression using a scoring system between 0 and 5 (Section 2.6.3) which is described in Table 2.5. Rectal temperatures were recorded daily until the rabbit died or was euthanased.

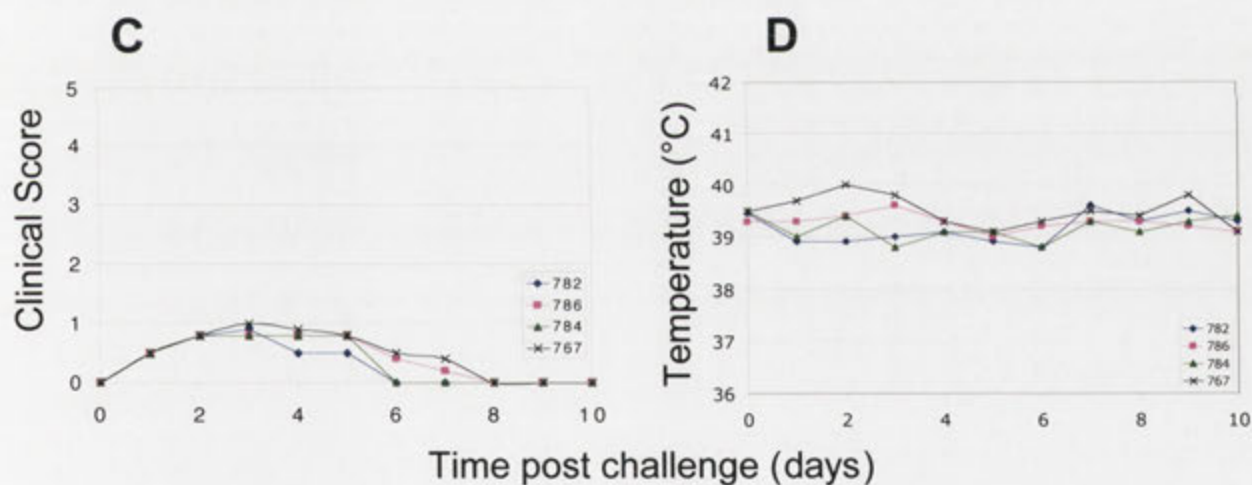
#### **3.2.7.1 *pDual2<sup>+</sup>*-vaccinated animals (negative controls)**

Rabbits vaccinated with the vector alone were treated as negative controls. It was expected that delivery of plasmid alone should not alter the progression of disease in these animals after challenge with SLS. All three control animals developed severe myxomatosis. The clinical scores of challenged animals began to increase from 2 dpi (Figure 3.10A) when a reaction at the inoculation site was generally visible. By 4 dpi, reddening of the conjunctiva was visible on two animals followed by mucopurulent discharge and hot, swollen ears by 6 dpi. Symptoms rapidly progressed until 8 dpi when all three animals had closed eyes with severe mucopurulent discharge, some discharge from the nose, anogenital oedema (AGO) and secondary lesions developing on the face and body. Two animals (857 and 847) displayed laboured and snuffly breathing (CS > 4). The control animals were euthanased at 9 dpi.

### pDual2+ vaccinated (negative controls)



### Uriarra-HA vaccinated (immune controls)



**Figure 3.10** - Clinical scores and rectal temperatures of rabbits vaccinated with pDual2+ (negative control) or Uriarra-HA (positive control; immune) after challenge with 1000 pfu of SLS. Clinical scores (A and C) and rectal temperatures (B and D) were recorded daily for rabbits vaccinated with pDual2+ or Uriarra-HA.

Rectal temperatures of pDual2+-vaccinated rabbits began to increase from 5 dpi, and were at 40°C or greater for all animals at 7, 8 and 9 dpi (Figure 3.10B).

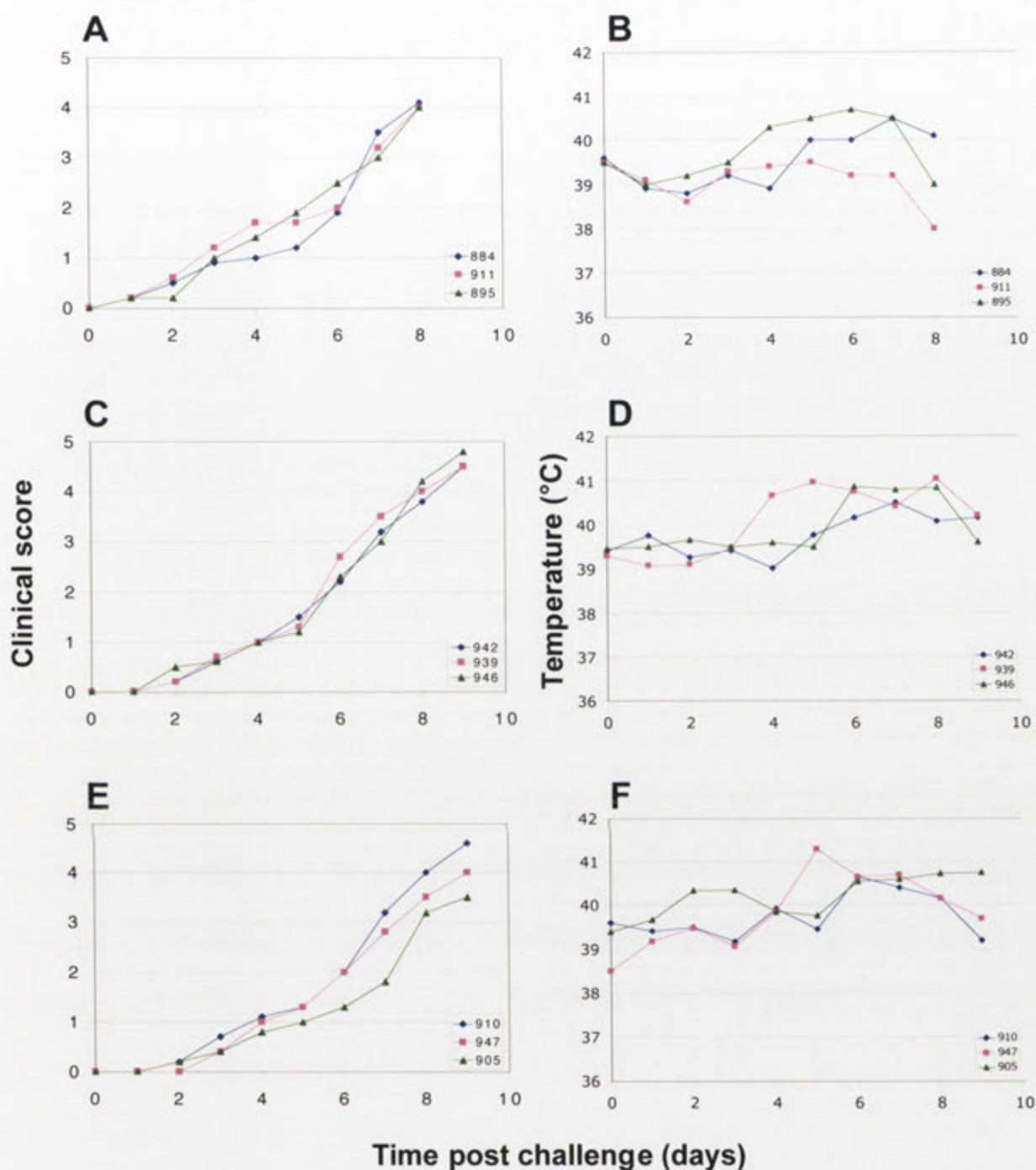
#### 3.2.7.2 Uriarra-HA-vaccinated (immune) animals

To observe the clinical signs of myxoma virus-immune rabbits following challenge with SLS, rabbits were first inoculated with the highly attenuated myxoma virus strain Uriarra-HA. This virus confers complete protection from myxomatosis following a very mild infection, generally limited to a primary lesion with secondary lesions developing in some animals (Kerr and Jackson, 1995; Kerr *et al.*, 2004). This group of animals was vaccinated with 1000 pfu of Uriarra-HA, and challenged with SLS three weeks later. Upon challenge with SLS, rabbits developed a small red spot at the inoculation site within 1 dpi, and some rabbits developed a small, localised primary lesion, no larger than 30 mm in size. This lesion scabbed over, regressed, and by 8 dpi was a flat scab with little to no inflammation. None of the challenged animals developed secondary lesions or any symptoms beyond a lesion at the inoculation site (CS < 1) (Figure 3.10C). Immune animals did not display sustained increases in rectal temperature following challenge (Figure 3.10D), although rabbit 767 had a temperature of 40°C at 2 dpi only.

#### 3.2.7.3 M055R-vaccinated animals

The post-challenge clinical scores for M055R-vaccinated rabbits are shown in Figure 3.11. Clinical scores and disease progression did not appear to vary from pDual2+-vaccinated controls, and all animals were euthanased by 9 dpi.

The rectal temperatures of challenged animals are shown in Figure 3.11. Most animals showed elevated temperatures (>40°C) between 6 and 8 dpi. One pDual-55R-vaccinated



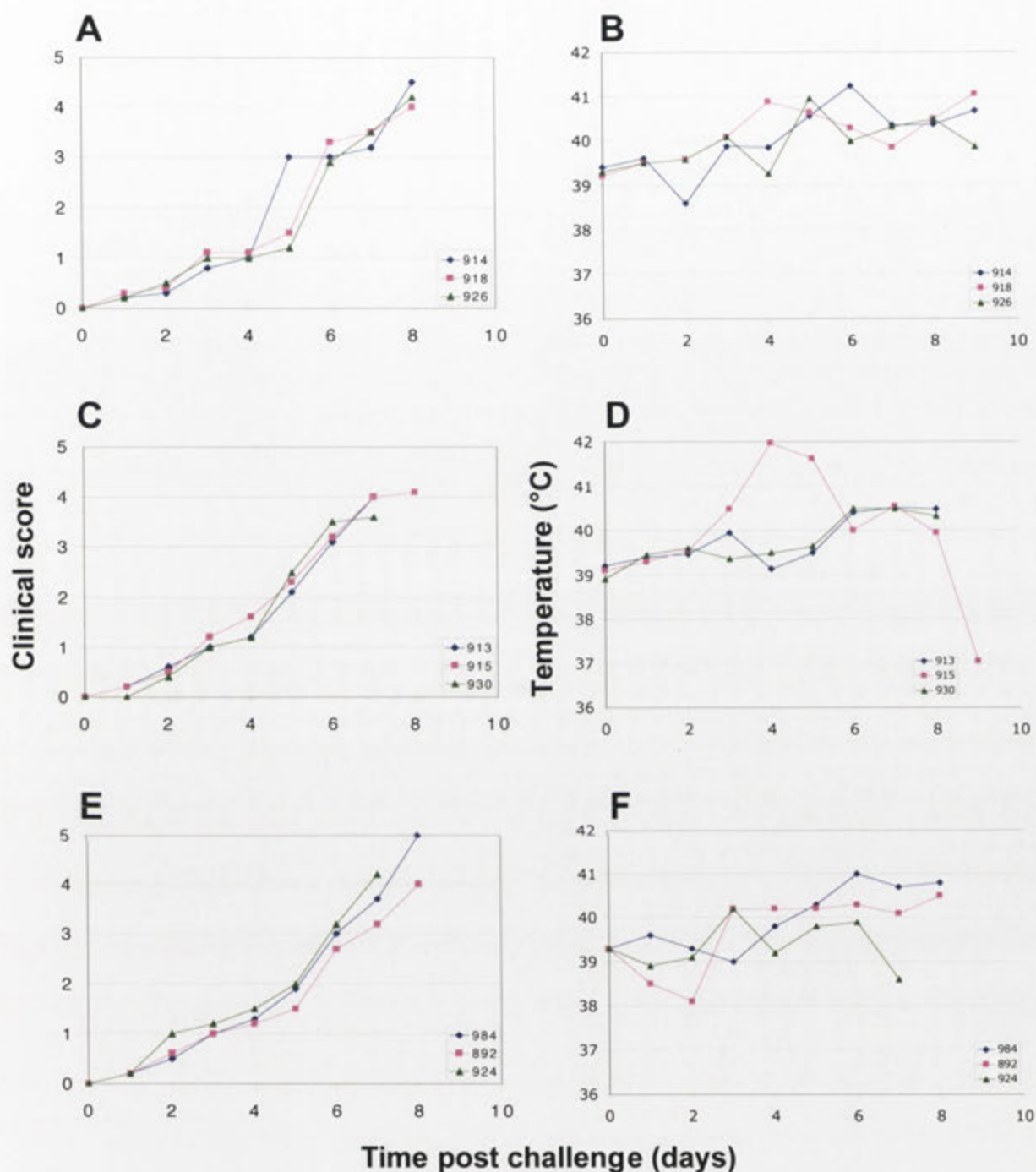
**Figure 3.11** - Clinical scores and rectal temperatures of rabbits vaccinated with M055R-expressing DNA vaccines after challenge with 1000 pfu of SLS. Each group of three rabbits vaccinated with pDual-55R (A and B), pDual-55R-IL2 (C and D) or pDual-55R-IL4 (E and F) was observed daily. The degree of disease progression for each animal was recorded as a clinical score between 0 and 5 (A, C and E). Rectal temperatures were recorded daily (B, D and F). The final data point indicates the animal was euthanased or was found dead on the following day.

animal (911) did not show an elevated temperature following challenge. However, a drop in temperature to 38°C was observed at 8 dpi. Several other animals showed a drop in temperature on day 8 or 9 post challenge. In these challenge experiments, animals that displayed a rapid decrease in temperature were euthanased, as previous experience has shown that these rabbits rarely survive for a further day (Best and Kerr, 2000).

#### *3.2.7.4 M073R-vaccinated animals*

Clinical scores for M073R-vaccinated rabbits are shown in Figure 3.12. Disease progression in these animals was indistinguishable from pDual2+-vaccinated animals, with most animals being euthanased at 9 dpi. One pDual-73R-IL4-vaccinated rabbit (924) displayed extremely rapid disease progression with a very large primary lesion. The primary lesion was 35 mm in diameter by 3 dpi, growing to 70 mm by day 7. The animal had facial lesions, closed eyes, extremely swollen ears and laboured breathing by 7 dpi, and was found dead on day 8. This case was the most severe manifestation of myxomatosis observed in all 52 challenged animals during the course of this experiment.

Post-challenge rectal temperatures of M073R-vaccinated rabbits are shown in Figure 3.12. Again, the profiles are more similar to pDual2+-vaccinated rabbits than immune controls, with most animals having a temperature of 40°C or greater from 5 or 6 dpi until death. A pDual-73R-IL2-vaccinated rabbit (915) displayed a substantial increase in temperature, reaching above 41°C on days 4 and 5 post infection. On day 9, the rabbit showed a drop in temperature to 37°C (2.5°C below normal), at which point it was euthanased.



**Figure 3.12 - Clinical scores and rectal temperatures of rabbits vaccinated with M073R-expressing DNA vaccines after challenge with 1000 pfu of SLS.** Each group of three rabbits vaccinated with pDual-73R (A and B), pDual-73R-IL2 (C and D) or pDual-73R-IL4 (E and F) was observed daily. The degree of disease progression for each animal was recorded as a clinical score between 0 and 5 (A, C and E). Rectal temperatures were recorded daily (B, D and F). The final data point indicates the animal was euthanased or was found dead on the following day.

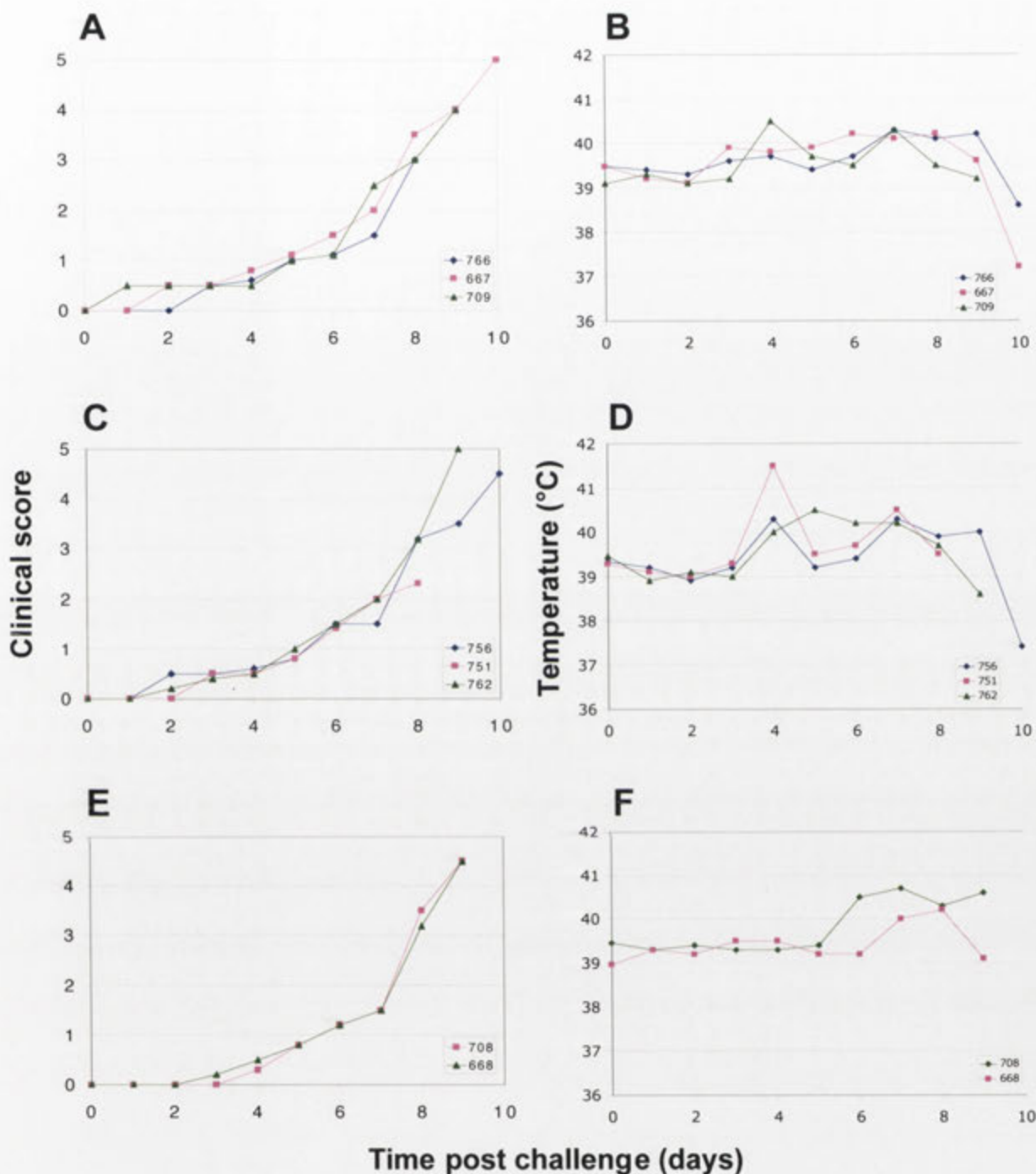


### 3.2.7.5 M115L-vaccinated animals

Clinical scores for challenged M115L-vaccinated rabbits are shown in Figure 3.13. There is some suggestion that clinical signs were slightly delayed in M115L-vaccinated animals with respect to pDual2+-vaccinated control animals (Figure 3.13). However, it is important to note that the clinical score data were collected by another researcher for these challenge experiments, which may have affected the scoring of the animals slightly due to emphasis of various symptoms, or different diagnosis of the severity of symptoms. Any suggestion of protection is eliminated by 8 dpi when the symptoms of pDual2+- and M115L-vaccinated rabbits were indistinguishable. Both sets of animals displayed laboured breathing, severe anogenital oedema, closed eyes and facial lesions.

One rabbit (705; pDual-115L-IL4-vaccinated) died a few days post-challenge from other causes, possibly an unrelated enteric infection. At the time of death, no clinical signs were visible. The data for this animal have not been included in Figure 3.13. The other two rabbits in the same challenge group exhibited disease progression similar to other M115L-vaccinated rabbits.

Most M115L-vaccinated rabbits had increased rectal temperatures later during the course of challenge compared to rabbits vaccinated with pDual2+ (Figure 3.13). This temperature increase more often occurred by 7 dpi, although pDual-115L-IL2-vaccinated animals had temperatures of 40°C or greater at 4 dpi. Again, several rabbits displayed a large drop in temperature to below 39°C on the final day (766, 667, 756 and 762) at which point the animals were euthanased.



**Figure 3.13 - Clinical scores and rectal temperatures of rabbits vaccinated with M115L-expressing DNA vaccines after challenge with 1000 pfu of SLS.** Each group of three rabbits vaccinated with pDual-115L (A and B), pDual-115L-IL2 (C and D) or pDual-115L-IL4 (E and F) was observed daily. The degree of disease progression for each animal was recorded as a clinical score between 0 and 5 (A, C and E). Rectal temperatures were recorded daily (B, D and F). The final data point indicates the animal was euthanased or was found dead on the following day.

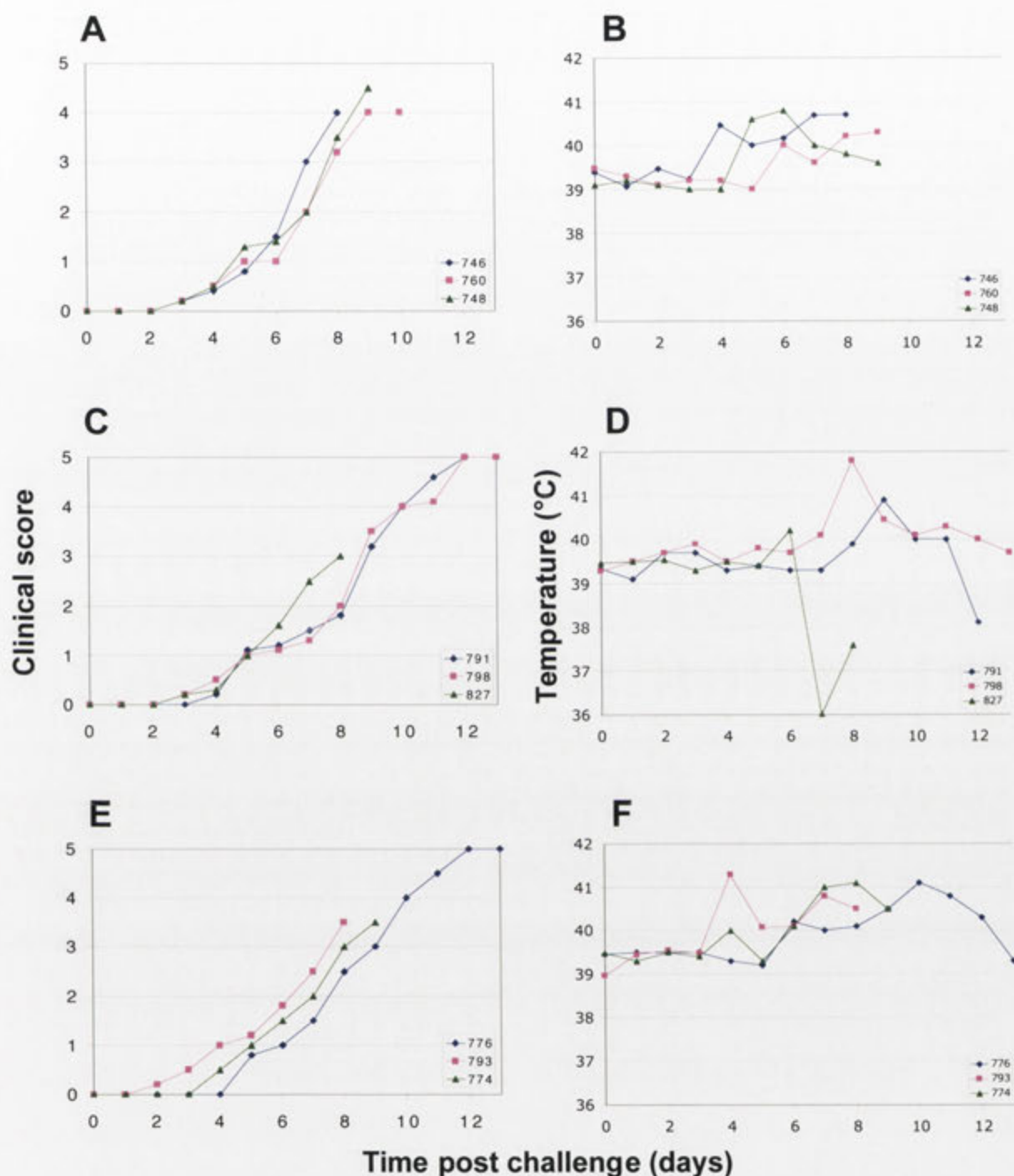
#### 3.2.7.6 *M121R-vaccinated animals*

The clinical signs of M121R-vaccinated rabbits following SLS challenge are shown in Figure 3.14. Most M121R-vaccinated rabbits began to show a reaction at the inoculation site by 3 or 4 dpi, as opposed to animals vaccinated with pDual2+, which showed a reaction by 2 dpi (Figure 3.10A). There was the possibility that this group of animals did not receive 1000 pfu of virus due to the temporary storage of challenge virus used in this experiment at -20°C instead of -80°C. This may have resulted in a slight degradation of the virus and a lower amount of virus delivered at challenge and may explain why symptoms appeared a day later than controls. However, any difference is eliminated later in infection when all animals displayed severe symptoms of myxomatosis.

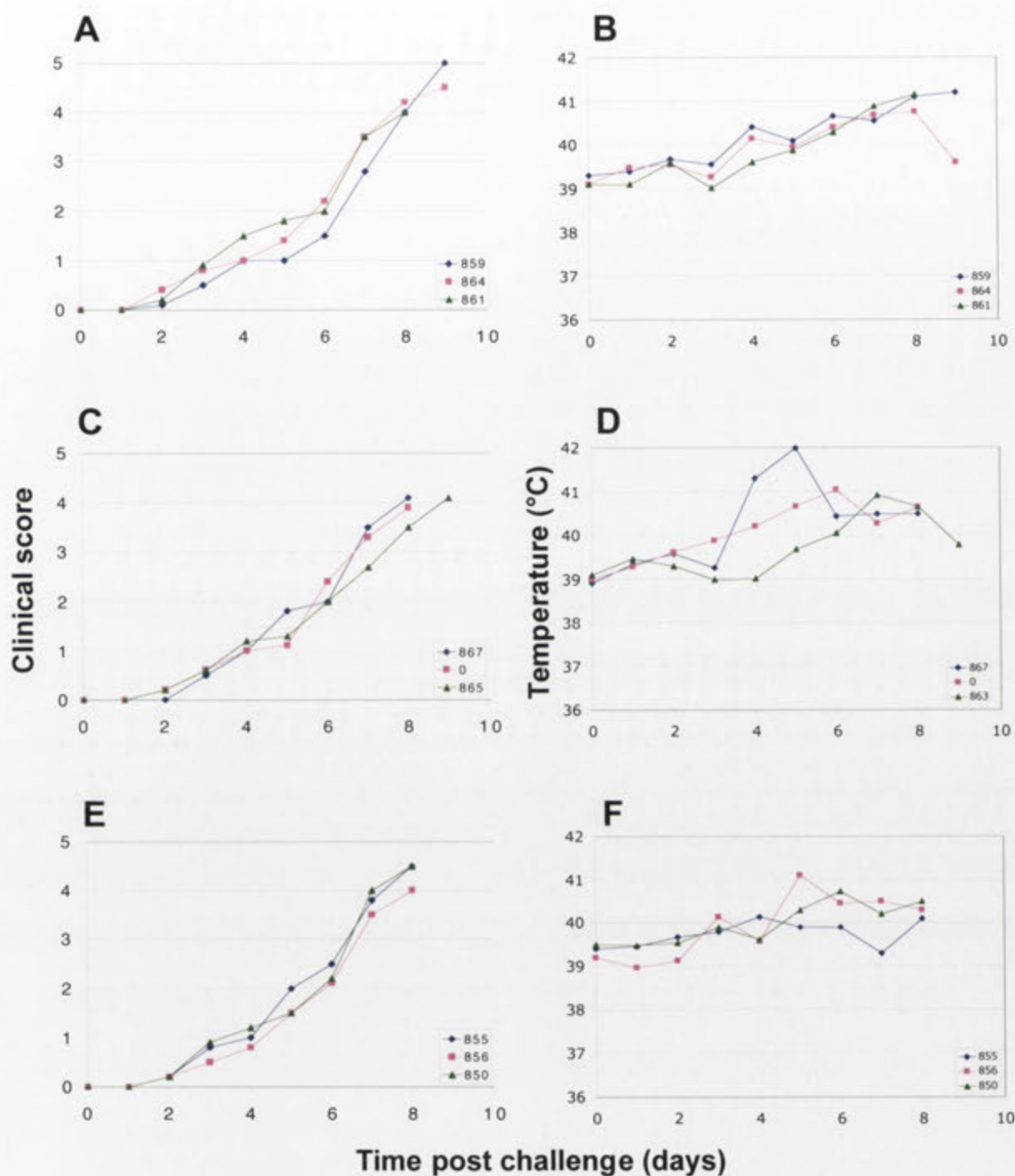
This group of animals was the first challenged in these DNA vaccination experiments. For this reason, they were not euthanased as early as other groups of animals, in case vaccination was able to offer some ability to control the infection. Three rabbits (791, 798 and 776) were not euthanased until 12 or 13 dpi, at which point all animals were extremely ill with laboured breathing. Rectal temperatures of M121R-vaccinated animals are shown in Figure 3.14. Many animals showed a fever with temperatures of 40°C or greater from 6 dpi until the end of the experiment.

#### 3.2.7.7 *M115L and M121R dual-vaccinated animals*

The co-administration of plasmid DNA expressing two myxoma virus antigens (M115L and M121R), and the delivery of twice as much plasmid DNA had no effect on the course of disease in rabbits challenged with SLS. Rabbits vaccinated with both M115L- and M121R-expressing vaccines showed similar clinical signs (Figure 3.15) to those vaccinated with the vector pDual2+ (Figure 3.10A). All challenged animals developed severe



**Figure 3.14 - Clinical scores and rectal temperatures of rabbits vaccinated with M121R-expressing DNA vaccines after challenge with 1000 pfu of SLS.** Each group of three rabbits vaccinated with pDual-121R (A and B), pDual-121R-IL2 (C and D) or pDual-121R-IL4 (E and F) was observed daily. The degree of disease progression for each animal was recorded as a clinical score between 0 and 5 (A, C and E). Rectal temperatures were recorded daily (B, D and F). The final data point indicates the animal was euthanased or was found dead on the following day.



**Figure 3.15 - Clinical scores and rectal temperatures of rabbits vaccinated with M115L- and M121R-expressing DNA vaccines after challenge with 1000 pfu of SLS.** Each group of three rabbits vaccinated with pDual-115L and pDual-121R (A and B), pDual-115L-IL2 and pDual-121R-IL2 (C and D) or pDual-115L-IL4 and pDual-121R-IL4 (E and F) was observed daily. The degree of disease progression for each animal was recorded as a clinical score between 0 and 5 (A, C and E). Rectal temperatures were recorded daily (B, D and F). The final data point indicates the animal was euthanased or was found dead on the following day.

myxomatosis and were euthanased 8 or 9 dpi. The post-challenge rectal temperatures of these animals also showed a similar profile to that observed in pDual2+-vaccinated rabbits (Figure 3.15).

### 3.2.8 Measurement of anti-myxoma virus immune responses following vaccination

Antigen-specific immune responses were measured one week after the final dose of plasmid for DNA-vaccinated animals, and 3 weeks following infection with Uriarra-HA for immune control animals. Antibody responses were qualitatively measured by either cellular immunofluorescence or immunoblotting, and cell-mediated responses were measured using a lymphocyte proliferation assay.

#### 3.2.8.1 Production of recombinant myxoma virus antigens for use in bioassays

The pET system (Novagen) was chosen for the production of recombinant myxoma virus antigens in the *E. coli* strain BL21(DE3) using the T7lac promoter system (Novagen, 2003; Studier *et al.*, 1990). The plasmid used for the cloning of the antigen genes, pET30a+, contains a series of purification and identification tags which can be expressed at the N-terminal end of the antigens. The genes coding for each of the antigens were cloned into pET30a+ in frame with the 5' sequence including the 6xHis tag that was used for later purification. This was achieved by removing the antigen gene from the pDual-Antigen plasmids as a restriction fragment and ligating it into pET30a+. The correct insertion and nucleotide sequence of the antigen genes were confirmed by sequencing. The plasmids were given the names pET-55R, pET-73R, pET-115L and pET-121R.

After propagation in *E. coli* DH5 $\alpha$ , the plasmids were transformed into the producer strain *E. coli* BL21(DE3), antigen production induced and antigens purified as described in

Section 2.3. After elution, individual fractions were screened for the presence of the antigen by SDS-PAGE. Fractions containing protein were pooled, concentrated and resuspended in PBS. The concentration of each antigen sample was calculated by comparison against Benchmark protein ladder (Invitrogen, USA). The recombinant proteins were diluted to 25 µg/mL with sterile PBS, and stored at -80°C.

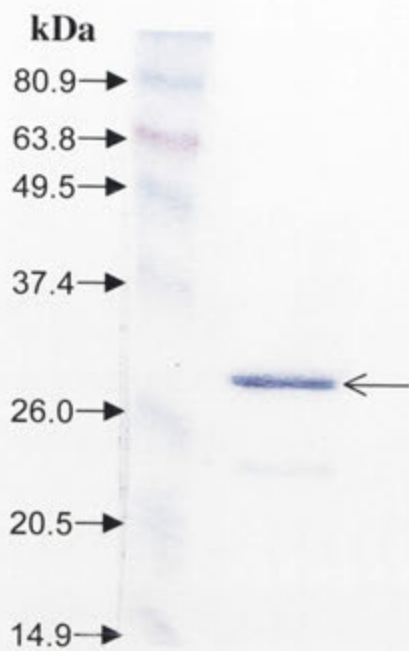
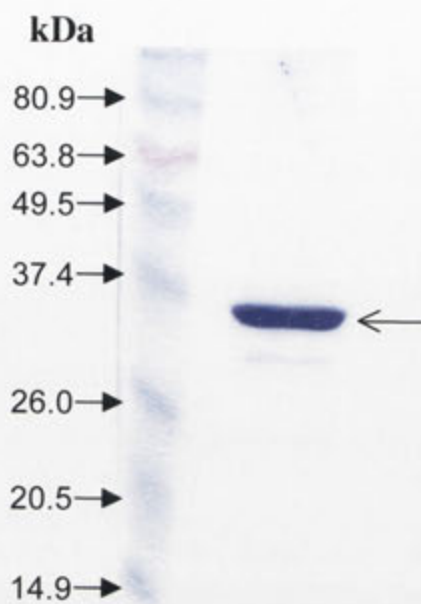
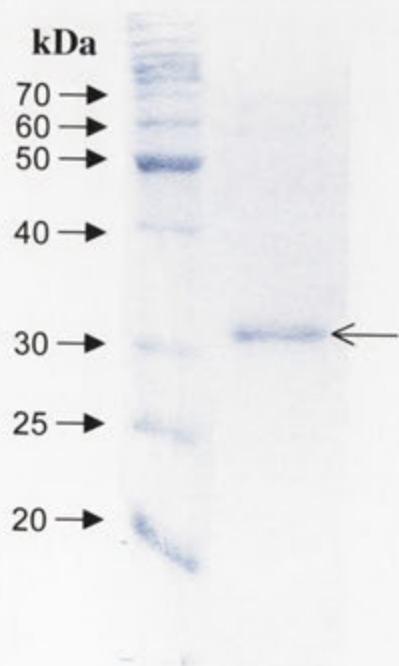
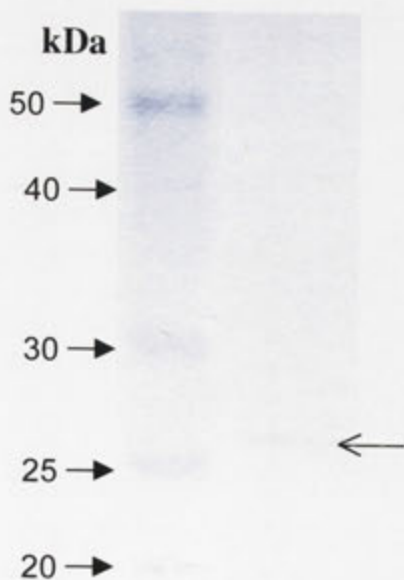
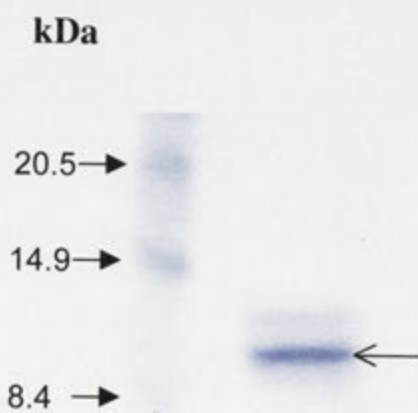
Protein purified from bacteria will contain some other proteins or compounds that may affect the *in vitro* PBMC proliferation assay. For this reason, the use of PBS as a negative control for the assay when using *E. coli*-produced antigen would not be suitable. Instead, *E. coli* BL21(DE3) was transformed with the vector pET30a+ only. When induced with IPTG, this plasmid codes for a 74 amino acid peptide containing all of the residues preceding the antigens, including the 6xHis tag, with a molecular weight of approximately 8.1 kDa. This protein, named rpET, was prepared and purified in the same way as the other antigens and was used as a negative control antigen in lymphocyte proliferation assays.

Figure 3.16 shows samples of eluted fractions for each recombinant antigen on Coomassie Blue-stained SDS-PAGE gels. The expected sizes for the recombinant antigens, including the addition of the purification tags to the N-terminal region of the protein were 31.9, 27.2, 27.3 and 25.4 kDa for rM055R, rM073R, rM115L and rM121R respectively. The actual sizes were approximately 31, 33, 31 and 26 kDa. The control antigen, rpET, was expected to have a molecular weight of 8.1kDa, but appears to be approximately 10 kDa in size, slightly larger than expected (Figure 3.16). Although the rM073R and rM115L bands were larger than expected, they were found to specifically bind with the polyclonal anti-myxoma virus serum on an immunoblot (data not shown), indicating that the bands do correspond to myxoma virus antigens. The other antigens, rM055R and rM121R were not detectable on

**Figure 3.16 - Purification of recombinant myxoma virus antigen proteins.** The genes encoding each of the myxoma virus antigens used in DNA vaccination experiments were cloned into the pET30a+ plasmid and protein production induced in *E. coli* BL21(DE3). After purification through TALON IMAC resin, protein (arrowed) was obtained for each antigen: M055R (A), M073R (B), M115L (C) and M121R (D). The protein production protocol was repeated using an *E. coli* strain containing the pET30a+ vector only as a negative control, producing a 10kDa protein (arrowed) containing only the N-terminal purification tags (E).

Each of A, B, C, D and E show the coloured protein markers (Benchmark Pre-Stained Protein ladder) in the left lane and the Coomassie Blue-stained gel containing the purified antigen protein in the right lane.



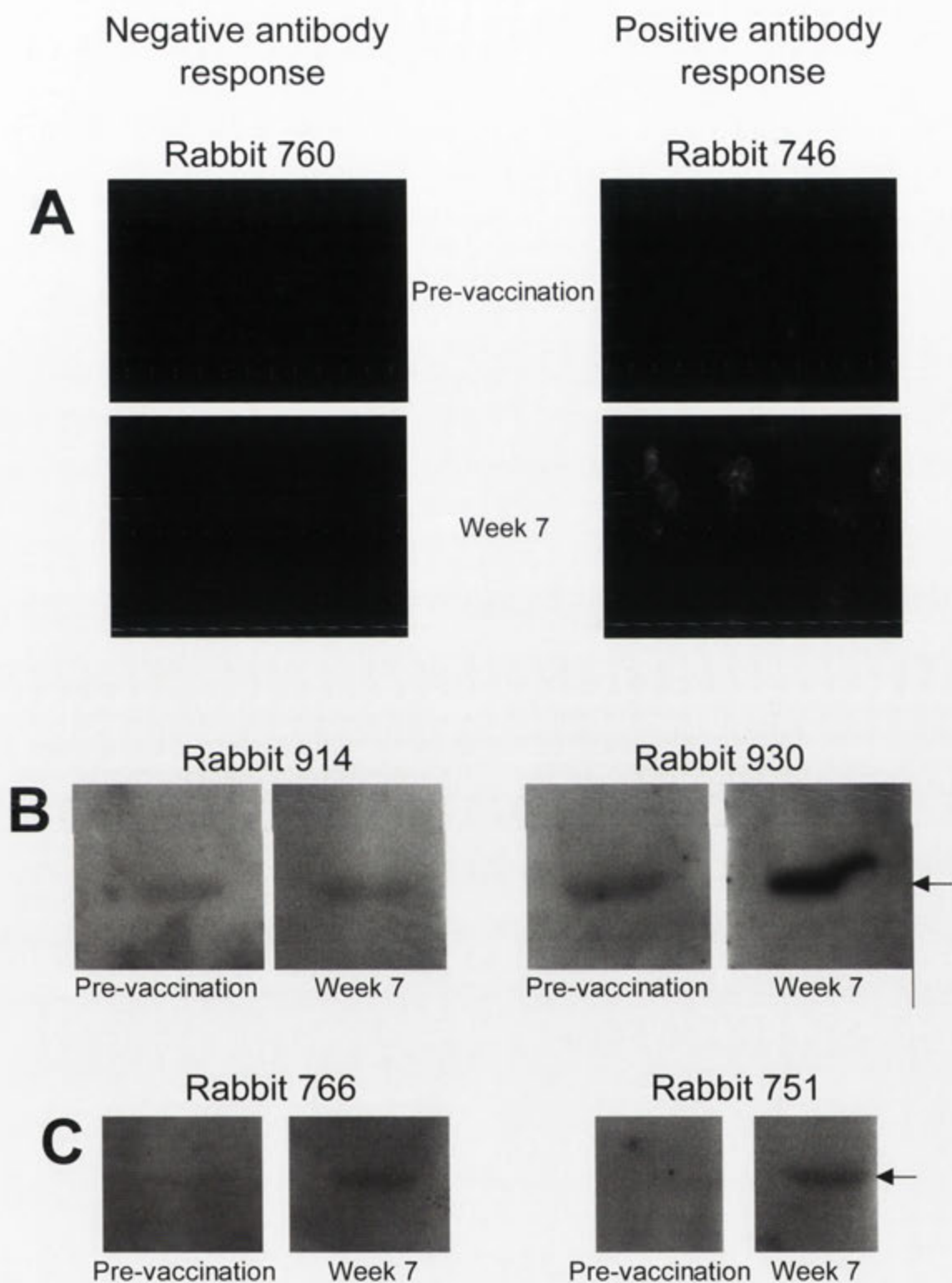
**A****B****C****D****E**

an immunoblot using the polyclonal anti-myxoma virus serum, as was found previously using antigen extracted from vaccine-transfected cells (Section 3.2.3.2). The yields obtained for each antigen varied significantly, as visible in Figure 3.16. Only low levels of rM121R were expressed, although enough protein was obtained for bioassays.

#### ***3.2.8.2 Measurement of antibody responses in DNA-vaccinated and control rabbits***

Anti-HA and anti-myxoma virus antibody responses were qualitatively measured by cellular immunofluorescence or immunoblotting. The detection of anti-HA, anti-M055R and anti-M121R antibodies was achieved by applying serum from a DNA-vaccinated rabbit (diluted 1:50) to RK13 cells transfected with pDual-HA, pDual-55R or pDual-121R respectively. An FITC-conjugated secondary antibody was then applied, and specific fluorescence observed. A positive antibody response was taken to be when there was an increase in the specific fluorescence observed on the transfected cell monolayer when serum collected after vaccination was compared to pre-vaccination serum. An example of a positive and a negative antibody response in M121R-vaccinated rabbits is displayed in Figure 3.17A.

Anti-M055R, anti-M073R and anti-M115L antibodies were detected by immunoblotting with recombinant myxoma virus antigens. A positive antibody response as measured by immunoblotting was determined as an increase in antibody binding to recombinant myxoma virus antigens on immunoblots of rM055R (anti-M055R responses), rM073R (anti-M073R responses) or rM115L (anti-M115L responses), when pre- and post-vaccination sera were compared. Examples of positive and negative antibody responses for M073R- and M115L-vaccinated rabbits are displayed in Figure 3.17B and C. Examples of



**Figure 3.17** - Examples of qualitative measurement of antibody responses in DNA-vaccinated rabbits. **A:** Anti-M121R antibody responses detected by cellular immunofluorescence on pDual-121R-transfected RK13 cell monolayers. **B:** Anti-M073R antibody responses detected by immunoblot using recombinant M073R protein (arrowed). **C:** Anti-M115L antibody responses detected by immunoblot using recombinant M115L protein (arrowed).

M055R-vaccinated rabbits have not been included as anti-M055R antibody was not detected in any serum sample using either immunoblotting or immunofluorescent methods.

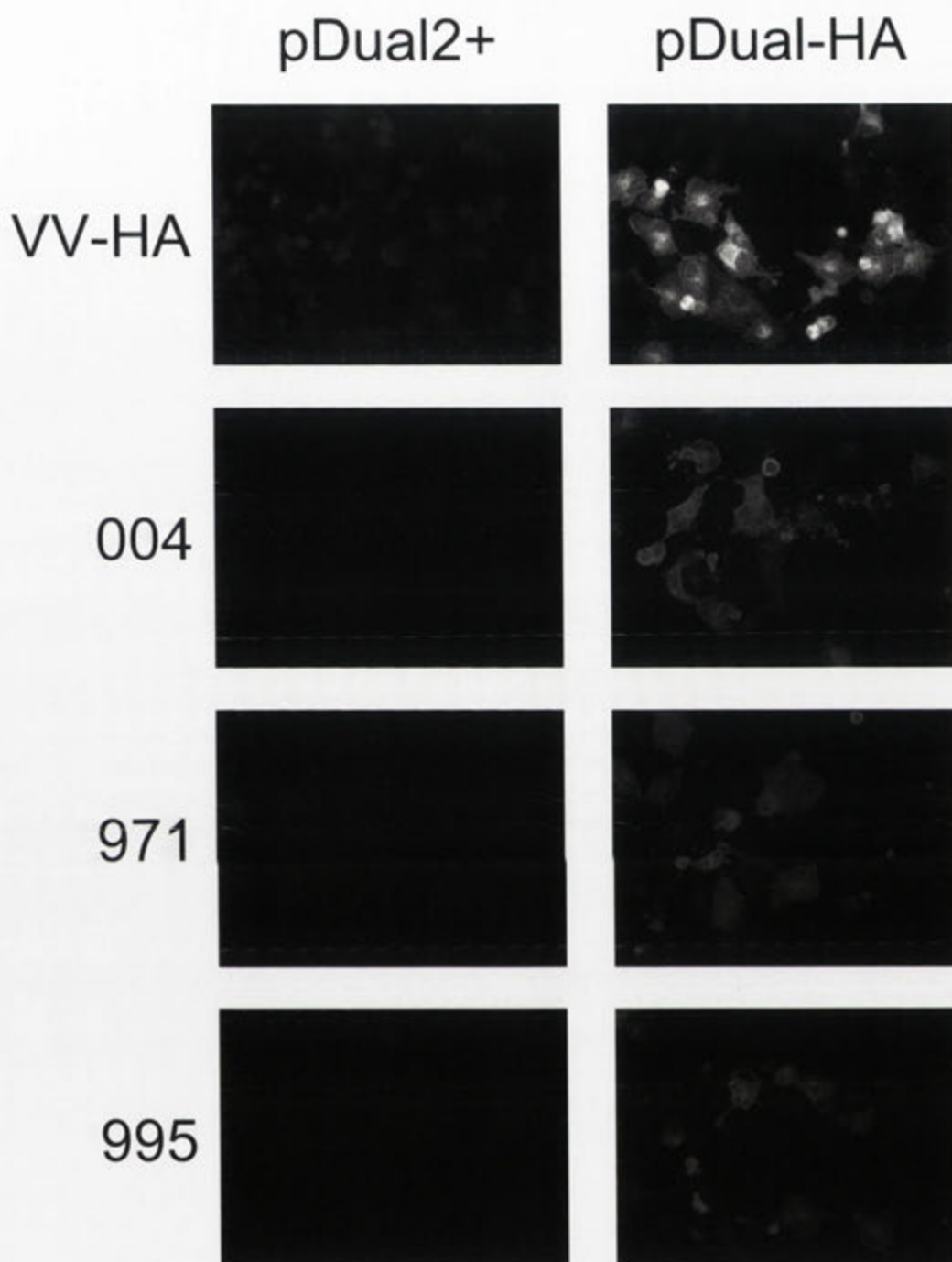
#### *3.2.8.2.1 pDual-HA vaccinated animals*

Anti-HA antibody responses were detected by cellular immunofluorescence. After 4 doses of pDual-HA, all three rabbits (004, 971 and 995) had readily detectable anti-HA antibodies, as observed by cellular immunofluorescence on pDual-HA-transfected cells (Figure 3.18). No immunofluorescence was observed on pDual2+ (vector only) transfected cells. The fluorescence intensity was lower than that observed when serum from a rabbit infected with VV-HA was applied to RK13 cells transfected with pDual-HA (Figure 3.18).

#### *3.2.8.2.2 Control animals*

Negative control animals were vaccinated with the vector pDual2+, following an identical vaccination regime as for the other DNA vaccines. The antigen-specific antibody responses were measured 1 week after the final dose of pDual2+. No myxoma virus-specific antibodies were detected in the sera collected from these rabbits (Table 3.3).

To determine the immune response to M055R, M073R, M115L and M121R in immune rabbits that had been infected and then recovered, six rabbits were vaccinated with Uriarra-HA (Kerr and Jackson, 1995). Antigen-specific antibody responses were measured 3 weeks after inoculation with virus. Sera from all immune animals showed readily detectable levels of anti-M121R, anti-M115L and anti-M073R antibodies (Table 3.3). However, no anti-M055R antibodies were detectable in sera from any immune animal, by either cellular immunofluorescence or immunoblotting.



**Figure 3.18** - Detection of anti-HA antibody responses in pDual-HA-vaccinated rabbits. Rabbits were immunised with vaccinia virus expressing influenza HA (VV-HA) or with pDual-HA (rabbit N° 004, 971 and 995) and serum collected 1 week after the final vaccine dose and applied to pDual2+ or pDual-HA-transfected RK13 cells.

**Table 3.3** – Antibody responses to individual myxoma virus antigens in negative control rabbits (pDual2+-vaccinated), immune rabbits (Uriarra-HA-vaccinated) and rabbits vaccinated with DNA vaccines expressing myxoma virus antigens.

Rabbit	Vaccine	Antibody response detected against <sup>a,b</sup>			
		M055R	M073R	M115L	M121R
846	pDual2+	-	-	-	-
847	pDual2+	-	-	-	-
852	pDual2+	-	-	-	-
767	Uriarra-HA	-	+	+	+
782	Uriarra-HA	-	+	+	+
784	Uriarra-HA	-	+	+	+
786	Uriarra-HA	-	+	+	+
884	pDual-55R	-			
911	pDual-55R	-			
895	pDual-55R	-			
942	pDual-55R-IL2	-			
939	pDual-55R-IL2	-			
946	pDual-55R-IL2	-			
910	pDual-55R-IL4	-			
947	pDual-55R-IL4	-			
905	pDual-55R-IL4	-			
914	pDual-73R		-		
918	pDual-73R		+		
926	pDual-73R		-		
913	pDual-73R-IL2		-		
915	pDual-73R-IL2		+		
930	pDual-73R-IL2		+		
984	pDual-73R-IL4		+		
892	pDual-73R-IL4		-		
924	pDual-73R-IL4		+		
766	pDual-115L			-	
667	pDual-115L			-	
709	pDual-115L			+	
756	pDual-115L-IL2			+	
751	pDual-115L-IL2			+	
762	pDual-115L-IL2			-	
708	pDual-115L-IL4			-	
668	pDual-115L-IL4			-	
705	pDual-115L-IL4			-	

Table 3.3 continued

Rabbit	Vaccine	Antibody response detected against <sup>a,b</sup>			
		M055R	M073R	M115L	M121R
746	pDual-121R				+
760	pDual-121R				-
748	pDual-121R				-
791	pDual-121R-IL2				-
798	pDual-121R-IL2				-
827	pDual-121R-IL2				+
776	pDual-121R-IL4				+
793	pDual-121R-IL4				-
774	pDual-121R-IL4				-
859	pDual-115L + pDual-121R			-	-
861	pDual-115L + pDual-121R			-	-
864	pDual-115L + pDual-121R			+	-
000	pDual-115L-IL2 + pDual-121R-IL2			-	-
865	pDual-115L-IL2 + pDual-121R-IL2			-	-
867	pDual-115L-IL2 + pDual-121R-IL2			-	-
850	pDual-115L-IL4 + pDual-121R-IL4			-	-
855	pDual-115L-IL4 + pDual-121R-IL4			+	+
856	pDual-115L-IL4 + pDual-121R-IL4			-	-

a: - : no detectable response; + : positive response.

b: An empty space indicates that responses against the antigen were not measured for the indicated rabbit.

#### 3.2.8.2.3 *Animals vaccinated with myxoma virus antigen-expressing plasmids*

The antigen-specific antibody responses of DNA-vaccinated rabbits are shown in Table 3.3. No M055R-vaccinated animals had anti-M055R antibodies detectable by either immunoblot or cellular immunofluorescence. Five M073R-vaccinated rabbits had positive anti-M073R antibody responses as determined by an increase in specific binding of antibody to the rM073R band when post- and pre-immune sera were compared on immunoblots (Figure 3.17). Three M115L-vaccinated animals showed the presence of anti-M115L antibodies as detected by immunoblotting. Serum from three M121R-vaccinated animals showed slightly increased binding to RK13 cells transfected with pDual-121R.

Only two rabbits that had been vaccinated with both M115L- and M121R-expressing vaccines possessed detectable anti-M115L antibody responses, and one of these animals (855) also had very low levels of anti-M121R antibodies (Table 3.3). Therefore, vaccination of rabbits with two antigens and twice as much plasmid DNA did not enhance antigen-specific antibody responses.

The magnitude of the antigen-specific antibody response against myxoma virus antigens in DNA-vaccinated rabbits was less than in Uriarra-HA-vaccinated rabbits. The degree of antibody binding to rM073R or rM115L on immunoblots from DNA-vaccinated rabbit sera was not as high as that observed from Uriarra-HA-vaccinated rabbit sera (data not shown). Similarly, fluorescence intensities observed when serum from DNA-vaccinated rabbits was applied to RK13 cells transfected with pDual-121R (Figure 3.17) were much weaker than those observed when serum from hyperimmune rabbits (Figure 3.2) or Uriarra-HA-infected (data not shown) rabbits was applied to the same cells.



There was no correlation between the co-expression of cytokines by DNA vaccines and the measured antigen-specific antibody responses. Of the 14 positive antibody responses recorded, four were from cytokine-free treatments, five from IL-2 treatments and five from IL-4 treatments.

### ***3.2.8.3 Measurement of PBMC proliferative responses in DNA-vaccinated and control rabbits***

Antigen-specific cell-mediated immune responses in vaccinated rabbits were measured by examining the antigen-specific proliferation of peripheral blood mononuclear cells (PBMC) isolated from rabbit blood 1 week after the final dose of vaccine plasmid, or 3 weeks after inoculation with Uriarra-HA for immune control animals. PBMC were pulsed with purified recombinant myxoma virus antigens produced in an *E. coli* expression system (Section 3.2.8.1). In Tables 3.4-3.10 are shown the measured counts per minute 5 days after addition of one of concanavalin A (as a positive control for T cell proliferation), PBS (as a negative control), the recombinant myxoma virus antigen or the negative control antigen rpET. Antigen-specific proliferative responses were recorded as a stimulation index (SI) calculated as the ratio between counts recorded in the presence of antigen and counts recorded in the presence of the negative control antigen (Section 2.7.1). The standard errors of counts were quite high with all treatments. Therefore, the statistical significance of each SI value was calculated using Fieller's theorem to determine the 95% confidence interval of the SI ratio (Finney, 1971). In Tables 3.4-3.10, a bold SI value indicates that the ratio was significantly greater than 1.5 ( $p < 0.05$ ), the value considered a positive proliferative response in these experiments.

#### 3.2.8.3.1 *pDual-HA-vaccinated animals*

A sample of influenza A/Puerto Rico/8 virus was obtained from Dr Sue McKenzie (CSIRO Sustainable Ecosystems). As this antigen was not produced in an *E. coli* expression system, PBS was used as a negative control. To confirm that the antigen sample did not induce non-specific responses in isolated rabbit PBMC, it was also added to PBMC isolated from unvaccinated rabbits.

PBMC isolated from rabbits vaccinated with pDual-HA proliferated in response to the addition of influenza A/Puerto Rico/8, with SI values of between 4.49 and 4.97 (Table 3.4), which were all significantly greater than 2.4. SI values from unvaccinated animals were approximately 1.0 (Table 3.4). The addition of concanavalin A (conA), a potent non-specific T cell mitogen, stimulated very high levels of PBMC proliferation (Table 3.4), with SI values of between 59 and 356 recorded. This indicates that PBMC isolated from rabbits were viable and capable of proliferation. Similar levels of PBMC proliferation in response to concanavalin A were found throughout the different groups of DNA-vaccinated rabbits.

These results, together with the antibody results shown in Figure 3.18, indicate that intramuscular injection of pDual2+ expressing a highly immunogenic viral antigen could induce cell-mediated and humoral immune responses in domestic rabbits.

#### 3.2.8.3.2 *Control animals*

The SI values recorded after addition of purified myxoma virus antigens to PBMC isolated from rabbits vaccinated with pDual2+ were low, ranging between 0.58 to 1.29 for all four viral antigens (Table 3.5). This indicated there was no antigen-specific proliferation of pDual2+-vaccinated rabbit PBMC in the presence of the four myxoma virus antigens.

**Table 3.4 – PBMC proliferation in response to influenza A/PR/8 stimulation in pDual-HA-vaccinated and unvaccinated animals.**

Rabbit	Vaccine	Proliferation <sup>a</sup> in Response to			Stimulation Index <sup>b</sup>
		Concanavalin A	Influenza A/PR/8 (1/50 dilution with PBS)	PBS	
004	pDual-HA	24523 ± 3673	701 ± 144	141 ± 20	<b>4.97</b>
971	pDual-HA	37095 ± 2868	515 ± 66	104 ± 27	<b>4.95</b>
995	pDual-HA	20182 ± 2414	355 ± 18	79.1 ± 34	<b>4.49</b>
181	Unvaccinated	18089 ± 2331	264 ± 27	304 ± 68	0.86
184	Unvaccinated	49857 ± 5952	416 ± 86	393 ± 57	1.06
187	Unvaccinated	13763 ± 8513	173 ± 76	171 ± 80	1.01

<sup>a</sup> Values represent mean cpm +/- S.E.M. of quadruplicate wells. Negative control is PBS alone.

<sup>b</sup> Stimulation Index = cpm of wells with antigen / cpm of wells with PBS. Figures in bold were significantly greater than 1.5 ( $p < 0.05$ ) as determined using Fieller's theorem.

**Table 3.5 – Proliferation of PBMC isolated from rabbits vaccinated with pDual2+ (negative control) or Uriarra-HA (immune; positive control) in response to recombinant M055R, M073R, M115L or M121R stimulation.**

Proliferation <sup>a</sup> in Response to										Stimulation Index <sup>b</sup>			
Rabbit	Vaccine	ConA	PBS	rM055R	rM073R	rM115L	rM121R	Negative control antigen (rpET)		M055R	M073R	M115L	M121R
846	pDual 2+	32752 ± 3721	819 ± 152	286 ± 60	322 ± 42	408 ± 204	334 ± 120	314 ± 71		0.91	1.02	1.29	1.06
847	pDual 2+	2619 ± 697	553 ± 130	331 ± 78	546 ± 240	465 ± 259	638 ± 440	568 ± 189		0.58	0.96	0.82	1.12
852	pDual 2+	173737 ± 15597	372 ± 133	250 ± 35	319 ± 38	314 ± 264	211 ± 12	375 ± 104		0.67	0.85	0.84	0.56
767	Uriarra-HA	361488 ± 13862	101945 ± 17316	N/D <sup>c</sup>	N/D <sup>c</sup>	85910 ± 31388	71739 ± 8209	88568 ± 12228				0.97	0.81
782	Uriarra-HA	280059 ± 12842	54363 ± 30322	N/D <sup>c</sup>	N/D <sup>c</sup>	28820 ± 12433	34563 ± 10803	81095 ± 17080				0.35	0.43
783	Uriarra-HA	108729 ± 5201	2252 ± 260	1952 ± 304	1894 ± 161	N/D <sup>c</sup>	N/D <sup>c</sup>	1073 ± 59		1.82	1.77		
784	Uriarra-HA	382969 ± 8139	71318 ± 15787	N/D <sup>c</sup>	N/D <sup>c</sup>	39230 ± 14609	88420 ± 21927	63132 ± 17868				0.62	1.40
786	Uriarra-HA	104031 ± 22230	3315 ± 891	N/D <sup>c</sup>	N/D <sup>c</sup>	2200 ± 382	1805 ± 369	886 ± 132		<b>2.15</b>		<b>2.48</b>	2.04
788	Uriarra-HA	13074 ± 1647	1621 ± 891	2607 ± 170	1856 ± 176	N/D <sup>c</sup>	N/D <sup>c</sup>	1211 ± 78			1.53		

<sup>a</sup> Values represent mean cpm +/- S.E.M. of quadruplicate wells. Negative control antigen is antigen purified from *E. coli* transfected with pET30a+ (rpET).

<sup>b</sup> Stimulation Index = cpm of wells with antigen / cpm of wells with negative control antigen. Figures in bold were significantly greater than 1.5 (*p* < 0.05) as determined using Fieller's theorem.

<sup>c</sup> N/D shows that the antigen was not tested with the indicated rabbit's lymphocytes.

PBMC were isolated from Uriarra-HA infected (immune) animals 3 weeks after inoculation with virus and stimulated with purified myxoma virus antigens. The SI values obtained for each animal and each antigen are recorded in Table 3.5. Some animals displayed a very high background level of lymphocyte proliferation regardless of the antigen added (rabbit numbers 767, 782 and 784). Similar counts were observed when cells obtained from these rabbits were pulsed with PBS alone (Table 3.5). Routine microscopic inspection of these cultures did not show any bacterial infection. This very high background proliferation may have masked any antigen-specific proliferation induced by the addition of myxoma virus antigens. Of the remaining immune animals, only responses against M055R and M115L were significantly greater than 1.5 for a single animal (Table 3.5).

#### *3.2.8.3.3 Animals vaccinated with myxoma virus antigen-expressing plasmids*

The antigen-specific proliferation of PBMC isolated from rabbits administered DNA vaccines expressing myxoma virus antigens were measured 1 week after the final dose of vaccine plasmid. None of the M055R-vaccinated rabbits had PBMC proliferative responses significantly greater than 1.5 (Table 3.6). Together with the antibody responses shown in Table 3.3, this suggests that DNA vaccination of rabbits with M055R did not result in a detectable anti-M055R immune response.

Two M073R-vaccinated rabbits (930 and 924) had SI values above 2.0, but only one pDual-73R-IL2-vaccinated rabbit (rabbit 930) had an SI significantly greater than 1.5 (Table 3.7). Both rabbits with SI values greater than 2.0 also had readily detectable anti-M073R antibodies (Table 3.3). However, other rabbits displaying positive anti-M073R antibody responses (rabbit numbers 918, 915 and 984) did not have significant levels of M073R-specific PBMC proliferation (Tables 3.3 and 3.7).

**Table 3.6 – Proliferation of PBMC isolated from rabbits vaccinated with M055R-expressing DNA vaccines in response to recombinant M055R stimulation.**

Proliferation <sup>a</sup> in Response to					Stimulation Index <sup>b</sup>
Rabbit	Vaccine	Concanavalin A	PBS	rM055R	Negative control antigen (rpET)
884	pDual-55R	34775 ± 4116	212 ± 13	264 ± 42	235 ± 46
911	pDual-55R	20744 ± 3831	232 ± 38	271 ± 22	226 ± 17
895	pDual-55R	2422 ± 412	344 ± 126	252 ± 34	167 ± 28
942	pDual-55R-IL2	77637 ± 412	158 ± 11	207 ± 8	244 ± 23
939	pDual-55R-IL2	23168 ± 3315	437 ± 175	566 ± 214	860 ± 129
946	pDual-55R-IL2	175651 ± 32731	1428 ± 306	2464 ± 261	1709 ± 824
910	pDual-55R-IL4	416 ± 75	267 ± 26	355 ± 52	730 ± 270
947	pDual-55R-IL4	104492 ± 4307	485 ± 122	1115 ± 474	936 ± 215
905	pDual-55R-IL4	4716 ± 934	542 ± 301	150 ± 29	191 ± 54

<sup>a</sup> Values represent mean cpm +/- S.E.M. of quadruplicate wells. Negative control antigen is antigen purified from *E. coli* transfected with pET30a+ (rpET).

<sup>b</sup> Stimulation Index = cpm of wells with antigen / cpm of wells with negative control antigen. Figures in bold were significantly greater than 1.5 (*p* < 0.05) as determined using Fieller's theorem.

**Table 3.7 – Proliferation of PBMC isolated from rabbits vaccinated with M073R-expressing DNA vaccines in response to recombinant M073R stimulation.**

Rabbit	Vaccine	Proliferation <sup>a</sup> in Response to			Stimulation Index <sup>b</sup>
		Concanavalin A	PBS	rM073R	
914	pDual-73R	113400 ± 11442	857 ± 59	1003 ± 336	1.88
918	pDual-73R	36294 ± 6138	884 ± 50	1460 ± 194	1.66
926	pDual-73R	38997 ± 9643	1255 ± 192	4731 ± 321	1.30
913	pDual-73R-IL2	43538 ± 3439	1198 ± 158	1318 ± 160	1.08
915	pDual-73R-IL2	33652 ± 3699	607 ± 60	378 ± 64	0.74
930	pDual-73R-IL2	10198 ± 2703	295 ± 77	601 ± 66	<b>2.51</b>
984	pDual-73R-IL4	3978 ± 1615	440 ± 75	209 ± 20	0.72
892	pDual-73R-IL4	99100 ± 20090	531 ± 227	401 ± 120	1.92
924	pDual-73R-IL4	50789 ± 9061	357 ± 35	562 ± 222	2.33

<sup>a</sup> Values represent mean cpm +/- S.E.M. of quadruplicate wells. Negative control antigen is antigen purified from *E. coli* transfected with pET30a+ (rpET).

<sup>b</sup> Stimulation Index = cpm of wells with antigen / cpm of wells with negative control antigen. Figures in bold were significantly greater than 1.5 ( $p < 0.05$ ) as determined using Fieller's theorem.

Of the nine rabbits vaccinated with M115L-expressing vaccines, two showed a proliferative response significantly greater than 1.5, and had SI values in excess of 4.0 (Table 3.8). There was no correlation between rabbits with detectable cell-mediated and humoral responses as both rabbits had no detectable anti-M115L antibodies (Table 3.3).

Of the nine rabbits vaccinated with M121R-expressing vaccines, only one showed a positive proliferative response to recombinant M121R protein (rabbit 760; Table 3.9), with an SI value of 5.33. This value had a lower 95% confidence interval of 3.3, the highest SI value recorded in these experiments. This animal did not have detectable anti-M121R antibodies (Table 3.3). None of the animals vaccinated with both M115L- and M121R-expressing vaccines showed positive proliferative responses to either antigen.

As was found with antigen-specific antibody responses, there was no overall decrease or increase in the antigen-specific PBMC proliferative responses due to co-expression of rabbit IL-2 or IL-4 by myxoma virus DNA vaccines. Of the four SI values significantly greater than 1.5 obtained, two were from cytokine-free treatments, one from IL-2 treatments and one from IL-4 treatments (Tables 3.6-3.10).

### 3.3 DISCUSSION

The results presented in this chapter indicate that DNA vaccines expressing the myxoma virus antigens M055R, M073R, M115L and M121R, and co-expressing the cytokines IL-2 and IL-4, are unable to protect rabbits from lethal myxoma virus challenge. These results suggest that simple intramuscular DNA vaccination is unable to protect rabbits from a complex virus such as myxoma virus. These results also demonstrate that data obtained during vaccination experiments in the VV/mouse model cannot simply be extrapolated to



**Table 3.8 – Proliferation of PBMC isolated from rabbits vaccinated with M115L-expressing DNA vaccines in response to recombinant M115L stimulation.**

Proliferation <sup>a</sup> in Response to					Stimulation Index <sup>b</sup>
Rabbit	Vaccine	Concanavalin A	PBS	rM115L	Negative control antigen (rpET)
766	pDual-115L	137853 ± 2709	3319 ± 541	5377 ± 579	3599 ± 557
667	pDual-115L	1204 ± 210	264 ± 48	752 ± 102	177 ± 36
709	pDual-115L	4707 ± 401	455 ± 23	640 ± 118	349 ± 119
756	pDual-115L-IL2	11751 ± 455	5451 ± 1813	6615 ± 503	8990 ± 1260
751	pDual-115L-IL2	8346 ± 917	4129 ± 464	1507 ± 262	3841 ± 337
762	pDual-115L-IL2	6186 ± 1676	252 ± 90	240 ± 29	144 ± 20
708	pDual-115L-IL4	36947 ± 3680	844 ± 135	1197 ± 408	1636 ± 167
668	pDual-115L-IL4	88464 ± 6597	3816 ± 356	4145 ± 690	5637 ± 741
705	pDual-115L-IL4	3670 ± 1337	349 ± 75	892 ± 76	219 ± 64

<sup>a</sup> Values represent mean cpm +/- S.E.M. of quadruplicate wells. Negative control antigen is antigen purified from *E. coli* transfected with pET30a+ (rpET).

<sup>b</sup> Stimulation Index = cpm of wells with antigen / cpm of wells with negative control antigen. Figures in bold were significantly greater than 1.5 (*p* < 0.05) as determined using Fieller's theorem.

**Table 3.9** – Proliferation of PBMC isolated from rabbits vaccinated with M121R-expressing DNA vaccines in response to recombinant M121R stimulation.

Proliferation <sup>a</sup> in Response to					Stimulation Index <sup>b</sup>
Rabbit	Vaccine	Concanavalin A	PBS	rM121R	Negative control antigen (rpET)
746	pDual-121R	98593 ± 4518	778 ± 101	983 ± 78	770 ± 58
760	pDual-121R	146259 ± 1757	109 ± 10	907 ± 150	170 ± 25
748	pDual-121R	159610 ± 18074	708 ± 86	1126 ± 340	511 ± 89
791	pDual-121R-IL2	330852 ± 22777	2303 ± 597	1932 ± 800	1398 ± 67
798	pDual-121R-IL2	95743 ± 21649	516 ± 90	597 ± 248	641 ± 85
827	pDual-121R-IL2	220466 ± 21028	660 ± 230	238 ± 49	543 ± 30
776	pDual-121R-IL4	234434 ± 9075	2101 ± 885	1218 ± 483	1084 ± 119
793	pDual-121R-IL4	193215 ± 17952	614 ± 153	1673 ± 1117	2935 ± 1923
774	pDual-121R-IL4	308983 ± 13211	3222 ± 1991	1689 ± 383	956 ± 170

<sup>a</sup> Values represent mean cpm +/- S.E.M. of quadruplicate wells. Negative control antigen is antigen purified from *E. coli* transfected with pET30a+ (rpET).

<sup>b</sup> Stimulation Index = cpm of wells with antigen / cpm of wells with negative control antigen. Figures in bold were significantly greater than 1.5 (*p* < 0.05) as determined using Fieller's theorem.

**Table 3.10 – Proliferation of PBMC isolated from rabbits vaccinated with both M115L- and M121R-expressing DNA vaccines in response to recombinant M115L or M121R stimulation.**

Proliferation <sup>a</sup> in Response to							Stimulation Index <sup>b</sup>	
Rabbit	Vaccine	Concanavalin A	PBS	rM115L	rM121R	Negative control antigen (rpET)	M115L	M121R
859	pDual-115L & pDual-121R	21736 ± 3368	118 ± 13	178 ± 36	318 ± 68	178 ± 8	1.00	1.79
861	pDual-115L & pDual-121R	28761 ± 2230	193 ± 89	196 ± 69	480 ± 186	174 ± 49	1.12	2.76
864	pDual-115L & pDual-121R	48564 ± 7731	536 ± 30	664 ± 177	653 ± 20	902 ± 360	0.74	0.72
000	pDual-121R	72158 ± 4513	1453 ± 114	1870 ± 260	1891 ± 338	1926 ± 260	0.97	0.98
865	pDual-115L-IL2 & pDual-121R-IL2	36033 ± 2110	588 ± 116	748 ± 334	747 ± 288	265 ± 62	2.82	2.82
867	pDual-115L-IL2 & pDual-121R-IL2	50122 ± 10725	493 ± 159	391 ± 215	495 ± 111	240 ± 74	1.63	2.06
850	pDual-115L-IL4 & pDual-121R-IL4	136954 ± 14204	1742 ± 763	1087 ± 430	1327 ± 403	816 ± 355	1.33	1.63
855	pDual-115L-IL4 & pDual-121R-IL4	39743 ± 4915	756 ± 67	836 ± 83	1230 ± 219	1238 ± 71	0.68	0.99
856	pDual-115L-IL4 & pDual-121R-IL4	9383 ± 1230	1015 ± 70	970 ± 228	1982 ± 517	637 ± 111	1.52	3.11

<sup>a</sup> Values represent mean cpm +/- S.E.M. of quadruplicate wells. Negative control antigen is antigen purified from *E. coli* transfected with pET30a+ (rpET).

<sup>b</sup> Stimulation Index = cpm of wells with antigen / cpm of wells with negative control antigen. Figures in bold were significantly greater than 1.5 ( $p < 0.05$ ) as determined using Fieller's theorem.

other poxvirus models, and questions the validity of comparing the VV/mouse model with natural poxvirus disease models.

### **3.3.1 Selection of candidate antigens**

Myxoma virus, like all poxviruses contains well over 100 genes, many of which have unknown functions (Cameron *et al.*, 1999). The antigen-specific responses and protective antigens of poxviruses have not been studied intensively until recently, and only in the VV/mouse model (Demkiewicz *et al.*, 1992; Galmiche *et al.*, 1999; Hooper *et al.*, 2000; Hooper *et al.*, 2003; Lai *et al.*, 1991b; Pulford *et al.*, 2004), which was the basis for the selection of genes to investigate in these experiments. These genes were selected based on studies where they were able to protect mice from lethal challenge with VV, or were the targets of potent neutralising antibody.

In hindsight, M071L (VV H3L) and M144L (VV B5R) would have been better candidates to test rather than M073R (VV H5R) and M107L (VV A17L), as they had demonstrated ability to protect mice from challenge with VV. However, the confusion in the literature between the identity of H3L and H5R was not identified until vaccines expressing M073R had already been administered to rabbits. It is also important to note that what were considered the best two candidates – M115L and M121R – were not protective either alone or delivered together, and thus it is perhaps unlikely that pDual2+-based vaccines expressing M144L or M071L would prove more effective than either of these candidates.

### **3.3.2 Detection of myxoma virus antigen expression in RK13 cells**

Of the five selected myxoma virus antigens, the expression of three in RK13 cells was visualised using antibody-mediated methods (cellular immunofluorescence and

immunoblotting) using the polyclonal anti-myxoma virus serum. The expression of M121R was clearly visible by cellular immunofluorescence, but the same serum was unable to bind to M121R on a Western blot. It may be that the anti-M121R antibodies in the polyclonal serum can only bind native protein, and are unable to bind to SDS-denatured M121R protein. In contrast, anti-M115L antibodies in the polyclonal serum were able to bind to M115L on an immunoblot under both reducing and non-reducing conditions, but the fluorescence observed by cellular immunofluorescence was much lower than that seen on cells transfected with pDual-121R. M073R was only detected by immunoblotting, which is a more sensitive method of detecting antigen expression than cellular immunofluorescence, as the protein is concentrated as a discrete band during SDS-PAGE. Without a quantitative means of measuring the expression of antigens, it cannot be determined whether the four tested antigens are being expressed at the same level, or if the observed differences in intensity of cellular fluorescence and bands on immunoblots was due to different levels of antibodies in the polyclonal anti-myxoma virus serum.

M107L mRNA or protein expression was not detected. Although analysis of M107L's nucleotide sequence did not strongly suggest the presence of a splice-consensus sequence, the lack of detectable M107L mRNA indicates that expression was not occurring. If M107L gene does contain a splice-consensus sequence, this would lead to inappropriate splicing and degradation of M107L mRNA. There are very few reports available in the literature on lack of DNA vaccine antigen expression due to inappropriate splicing, most likely because this data is simply not published. However, Zhou *et al.*, (2002) found that cryptic splice sites in the *env* gene of equine infectious anaemia virus resulted in reduced immune responses to the antigen during DNA vaccination studies, which were overcome by site-directed mutagenesis of the cryptic splice site.

Expression of the VV homologue (A17L) under control of the CMV-IE promoter in a DNA vaccine could not be detected (Jay Hooper, USAMRIID, personal communication), suggesting that expression of M107L and its homologues in other poxviruses may be dependent on the presence of other viral antigens.

### **3.3.3 Measurement of antigen-specific immune responses**

The PBMC proliferation assay used in these experiments measured the presence of antigen-specific CD4<sup>+</sup> T cells in the blood of vaccinated animals (Han *et al.*, 1999b; Han *et al.*, 2000b). It would have been preferable to use an assay to measure CTL responses in vaccinated rabbits as CTLs are the effector cells believed to be responsible for clearance of and protection from myxoma virus (Kerr and McFadden, 2002). However, there is currently no assay to measure CTL activity in the myxoma virus model. The use of outbred rabbits in the vaccination experiments prevented the use of standard <sup>51</sup>Cr release assays to measure antigen-specific CTL responses as MHC-compatible cells lines were not available. Therefore, it was decided to use the lymphoproliferative assay to measure cell-mediated responses.

Recombinant myxoma virus antigens were used in this assay because whole virus would have would have resulted in the inhibition of any antigen-specific proliferation. The proliferation of rabbit lymphocytes is inhibited upon infection with myxoma virus, both before and after addition of T cell mitogens such as concanavalin A (Collins, 2004). This effect extends to inactivated virus, which can prevent cellular proliferation, possibly through signals following binding of cellular and viral receptors (Strayer *et al.*, 1983; Peter Kerr, personal communication).

The PBMC proliferation assay was quite ‘noisy’, in that high standard errors were recorded, often 10-30% of the mean values. These errors were recorded across all treatments including mitogen- and PBS-treated cells. This resulted in most SI values being statistically insignificant, as determined by Fieller's theorem. A similar level of noise has been recorded by others working with rabbit lymphocyte proliferation assays (Han *et al.*, 1999b; Han *et al.*, 2000b), and it appears that the use of rabbit cells in this assay is inherently much noisier than when using murine cells (Demkowicz *et al.*, 1992; Lai *et al.*, 1991b). Christensen *et al.*, from whom the rabbit PBMC proliferation protocol was obtained (Han *et al.*, 1999a; Han *et al.*, 1999b) have since used delayed-type hypersensitivity (DTH) tests to measure cell-mediated immune responses against antigens delivered as DNA vaccines, and have found better correlation between observed DTH responses and protective efficacy in the cottontail rabbit papilloma virus (CRPV) model (Han *et al.*, 2000a; Han *et al.*, 2000b; Hu *et al.*, 2002).

The immune responses measured against the viral antigens did not affect disease progression after challenge. Even animals with significant and strong SI values ( $SI > 4$ ) or higher antibody responses showed comparable disease progression to animals with no detectable responses. Similar poor correlation between measured immune responses and protective immunity have been noted in other rabbit DNA vaccination studies (Han *et al.*, 2000a; Han *et al.*, 1999b; Han *et al.*, 2000b). Studies in cancer immunotherapy have also shown poor correlation between the magnitude of measured cellular immune responses and protective efficacy (Bellone *et al.*, 1999). This is believed to be due to the fact that while low avidity T cells (T cell clones with a low binding strength between the TCR and the antigen peptide-loaded MHC molecule) are readily detected by *in vitro* assays, these cells have little biological activity *in vivo* where high avidity T cells are required (Gilboa, 1999).

### 3.3.4 Lack of cytokine efficacy

There was no observed enhancement of antigen-specific immune responses by co-expression of rabbit cytokine genes in this study. Based on previous DNA vaccination studies in other species (mice, humans, monkeys), enhanced antibody responses in IL-4 vaccinated rabbits and enhanced cellular responses in IL-2 vaccinated rabbits were expected (Pan *et al.*, 1999). The reason for this lack of cytokine effect is unknown. In other studies, cytokines have been delivered on a separate plasmid to the antigen. The co-expression of cytokines from the same plasmid using a bi-cistronic vector has resulted in similar results to separate plasmid delivery (Chow *et al.*, 1997), although separate cytokine-expressing plasmid delivery has also been shown to be more effective at eliciting CD4<sup>+</sup> T cell responses (Barouch *et al.*, 2002).

Murine GM-CSF has been used to enhance immune responses in DNA vaccinated rabbits using the CRPV model (Leachman *et al.*, 2000), but this was the first study where rabbit cytokine cDNAs have been used in a vaccination experiment. Recombinant myxoma viruses expressing rabbit IL-2 or IL-4 displayed enhanced virulence in rabbits (Junankar, 1999; Kerr *et al.*, 2004), suggesting that these cytokines have potent *in vivo* activity. In other animal models, IL-2 cDNA co-delivery has enhanced cell-mediated and antibody responses to plasmid-delivered antigens and skewed immune responses towards Th1-like responses (Pan *et al.*, 1999). Similarly, IL-4 co-expression by DNA vaccines has resulted in increased seroconversion rates and improved antibody titres to plasmid-delivered antigens, and a predominantly Th2-like immune response (Pan *et al.*, 1999).



### 3.3.5 DNA vaccines in rabbits and alternative DNA vaccination protocols

DNA vaccines have protected domestic rabbits from challenge with viruses in several different models including pseudorabies (Hong *et al.*, 2002), CRPV (Christensen *et al.*, 2001; Hu *et al.*, 2002; Leachman *et al.*, 2002) and rinderpest (Joshi *et al.*, 2002). This indicates that with the correct protective antigen, rabbits can be successfully DNA vaccinated against pathogenic viruses, even using intramuscular injection of plasmid (Hong *et al.*, 2002; Joshi *et al.*, 2002). Alternative delivery methods such as gene-gun inoculation may induce a protective immune response where intramuscular injection of plasmid DNA does not (Han *et al.*, 2000b).

Considerable work has been undertaken in mouse models to enhance the immune response to DNA immunogens. Tested variables include the location of the muscle in which the DNA is injected (Yokoyama *et al.*, 1997), injection volume (Dupuis *et al.*, 2000) and the use of various cytokine or immunomodulatory genes (Egan and Israel, 2002). More recent methods involve intradermal injection (Raz *et al.*, 1994), intralymphatic injection (Maloy *et al.*, 2001), electroporation of muscles following plasmid injection (Widera *et al.*, 2000), the use of lipid carriers to enhance cellular transfection (Fischer *et al.*, 2003) and the inclusion of immunostimulatory DNA sequences (CpG motifs) on the plasmid backbone or co-injected with the plasmid (Klinman *et al.*, 1997; Mutwiri *et al.*, 2003). Whether any of these methods would enhance the protective efficacy of the tested myxoma virus DNA vaccines is unknown. These tests and protocol modifications are generally performed once an effective candidate antigen has been selected, to enhance the immune responses generated by the DNA vaccine. The use of M055R, M073R, M115L or M121R in such an optimisation regime may still result in an infective vaccine if they are not key protective antigens for myxoma virus.

One important factor in this study may have been injection volume. Studies of protective vaccines in rabbits utilised smaller amounts of plasmid DNA, but in greater volumes (1–2 mL rather than 250  $\mu$ L; Hong *et al.*, 2002; Joshi *et al.*, 2002). Smaller volumes were utilised in this study as such large volume injections can cause significant discomfort to rabbits. However, the larger volume injection also enhances drainage of the plasmid solution from the injection site to the draining lymphatics where enhanced antigen presentation and induction of immunity can occur. Larger injection volumes have also resulted in enhanced transfection of muscle cells and immune stimulation, possibly due to higher hydrostatic pressure in the muscle bundle (Dupuis *et al.*, 2000). It was also suggested by Dupuis *et al.*, (2000) that intramuscular DNA vaccination in larger animals is not as effective as in mice due to the larger relative muscle to vaccine volume, resulting in a correspondingly lower hydrostatic pressure and rate of cellular transfection. A comparison of injection volumes in rabbits has not been performed.

### **3.3.6 Conclusions**

These sets of experiments demonstrate that an easily delivered plasmid DNA vaccine to protect rabbits from myxomatosis is not feasible at this point. Although alternative delivery methods or altered formulations may allow these constructed DNA vaccines to induce protective responses, many of these technologies are all still lab-based methods which are not currently available to veterinarians.

The further screening of other myxoma virus antigens may reveal a highly immunogenic, protective antigen. However, this approach is still high risk in that a single protective antigen may not exist, or it may be an antigen of unknown function with little evidence pointing to its identity. The large scale screening of many antigens would be time

consuming, expensive and may not yield an effective vaccine. Therefore, it was decided to investigate recombinant myxoma virus strains as potential non-transmissible myxomatosis vaccines. Attenuated poxvirus vaccines have been highly efficacious in protecting against poxvirus diseases in many different models (Aspden *et al.*, 2003; Fenner, 1993; Ngichabe *et al.*, 2002; Tripathy, 1999; Tulman *et al.*, 2002). In Chapter 4, the construction and testing of several recombinant myxoma virus strains based on the Uriarra strain of myxoma virus will be described.

## **Chapter 4**

### **Construction and testing of myxoma virus deletion mutants as candidate vaccines**

## **CHAPTER 4 - CONSTRUCTION AND TESTING OF MYXOMA VIRUS DELETION MUTANTS AS CANDIDATE VACCINES**

### **4.1 INTRODUCTION**

The effectiveness of live virus vaccines to protect against poxvirus diseases has been well established in many different poxvirus disease models, and attenuated vaccines are routinely used to protect humans and livestock from diseases such as scabby mouth disease, goatpox, smallpox and lumpy skin disease (Esposito and Fenner, 2002). As described in Section 1.5, live attenuated myxoma virus vaccines, including the antigenically related poxvirus, rabbit fibroma virus (RFV) and tissue culture-attenuated strains of myxoma virus, are used to protect domestic rabbits against myxomatosis. These vaccines are used in Europe with varying degrees of success (Marlier *et al.*, 2000).

This chapter describes the construction of new candidate vaccine strains by the deletion of one or more key immunomodulatory genes from Uriarra. This virus was isolated from wild rabbits in the Canberra region of Australia in 1953 (Mykytowycz, 1953), and the current strain has since been plaque purified (Russell and Robbins, 1989). This virus is of Grade V virulence, with only 5% lethality in domestic rabbits, although all rabbits develop moderate to severe symptoms of myxomatosis upon infection (Best and Kerr, 2000).

The genes chosen for deletion were selected based on the following criteria:

1. Their role in promoting dissemination of the virus *in vivo*. Their deletion would reduce or eliminate the appearance of secondary signs of myxomatosis and secondary lesions which could be a site of arthropod-borne transmission of the vaccine virus;

2. Their role in lesion growth and development. Their deletion would reduce the size of the primary lesion, and therefore the potential area on the animal from which the virus could transmit;
3. Their role in suppression of cell-mediated immune responses. Their deletion would enhance the host's response to the vaccine virus and produce an infection that is rapidly controlled, in that virus titres in the skin do not reach transmissible titres.

The three genes selected for deletion were M010L, M011L and M007. M011L is a key inhibitor of apoptosis in infected lymphocytes (Macen *et al.*, 1996), and M010L encodes an epidermal growth factor homologue, which plays a key role in lesion growth and development (McFadden *et al.*, 1995). Deletion of these genes inhibited dissemination of the virus to secondary sites in the animal, and the development of the primary and secondary lesions (Opgenorth *et al.*, 1992). The M010L/M011L genes, due to their overlapping open reading frames (ORFs) could be deleted in a single recombination event.

M007 encodes the myxoma virus  $\gamma$ -interferon receptor homologue M-T7, a key virulence factor of myxoma virus (Mossman *et al.*, 1996b). M-T7 also binds to CC, C and CXC chemokines (Lalani *et al.*, 1997). Deletion of M-T7 from the Lausanne strain of myxoma virus prevented the interruption of cellular immune responses, not only at the sites of viral replication, but in distal lymphoid organs (Mossman *et al.*, 1996b). The overall effect was an enhanced cellular immune response against the virus, more efficient migration of immune cells into sites of infection and much more rapid clearance of the virus.

It was postulated that in a vaccine, deletion of these genes would result in a much more rapid and robust anti-myxoma virus immune response which would reduce the titre of virus

at sites of infection and result in more rapid clearance, lowering the titre of virus in the skin below transmissible levels.

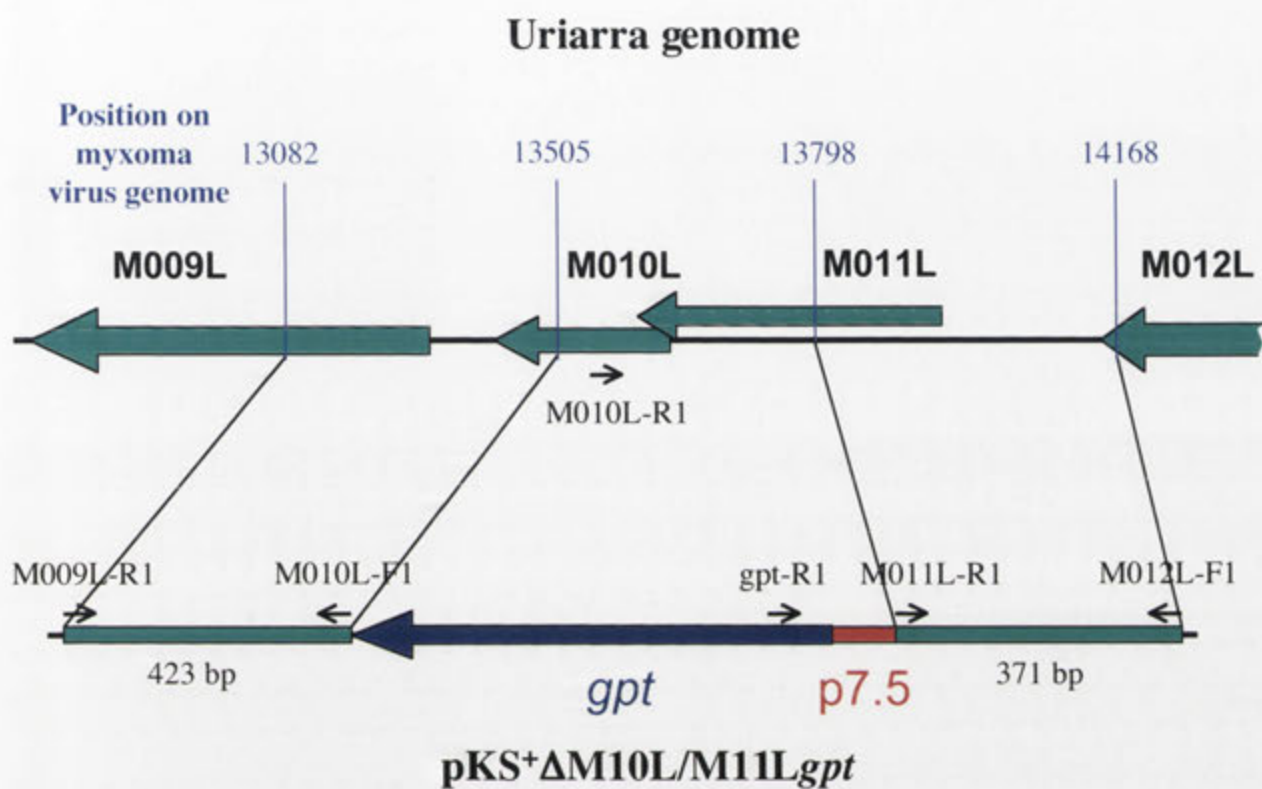
This chapter deals with the construction, purification and testing of three myxoma virus candidate vaccines. The vaccines were evaluated for two characteristics – acceptability and efficacy. The acceptability of the vaccine to pet rabbit owners was gauged by the severity of the symptoms caused by the vaccine virus, especially considering the appearance of secondary symptoms such as secondary lesions on the face and ears, which would not be acceptable in a companion animal. The protective efficacy of the vaccines was evaluated as the ability to protect rabbits from a lethal myxoma virus challenge. Candidate vaccine strains that were considered acceptable and effective were then selected for further analysis.

## **4.2 RESULTS**

### **4.2.1 Construction of recombinant myxoma virus vaccines**

#### *4.2.1.1 Design and construction of pKS<sup>+</sup>ΔM10L/M11Lgpt and Uriarra ΔM10L/M11Lgpt*

The inactivation of the overlapping ORFs of M010L and M011L was achieved by homologous recombination in Uriarra infected RK13 cells with the plasmid pKS<sup>+</sup>ΔM10L/M11Lgpt (Figure 4.1). This plasmid contains two regions of sequence homologous to the Uriarra genome, between which was cloned a selective marker, the *E. coli* gene xanthine guanine phosphorybosyl transferase (*gpt*; Richardson *et al.*, 1983), under control of the poxvirus early/late promoter, p7.5 (Davidson and Moss, 1989). This method, first developed by Falkner and Moss (1988), is routinely used to generate recombinant poxviruses. A recombination event between the plasmid and Uriarra genomic DNA results in a deletion of 292 bp from the genome of Uriarra, including the first 148 bp of M010L (57.8% of the ORF) and the last 173 bp of M011L (34.7% of the ORF), and its replacement



**Figure 4.1 - Construction of plasmid for deletion of the M010L and M011L sequences of myxoma virus.** Short sequences (423 and 371 bp) homologous to Uriarra DNA were cloned by PCR using the primers (small black arrows; Table 2.3) indicated, between which the selective gene xanthine guanine phosphorybosyl transferase (*gpt*) under control of the poxvirus promoter p7.5, was cloned. Homologous recombination of this plasmid with the Uriarra genome results in a recombinant virus (Uriarra ΔM10L/M11L*gpt*) with a 292 bp deletion including the first 148 bp of M010L and last 173 bp of M011L. The positions of other primers used for the PCR screening of recombinant viruses (M010L-R1 and gpt-R1) are indicated. The position of the ends of the homologous sequences are indicated as described in the published myxoma virus sequence (Genbank AF170726). Figure is not to scale.

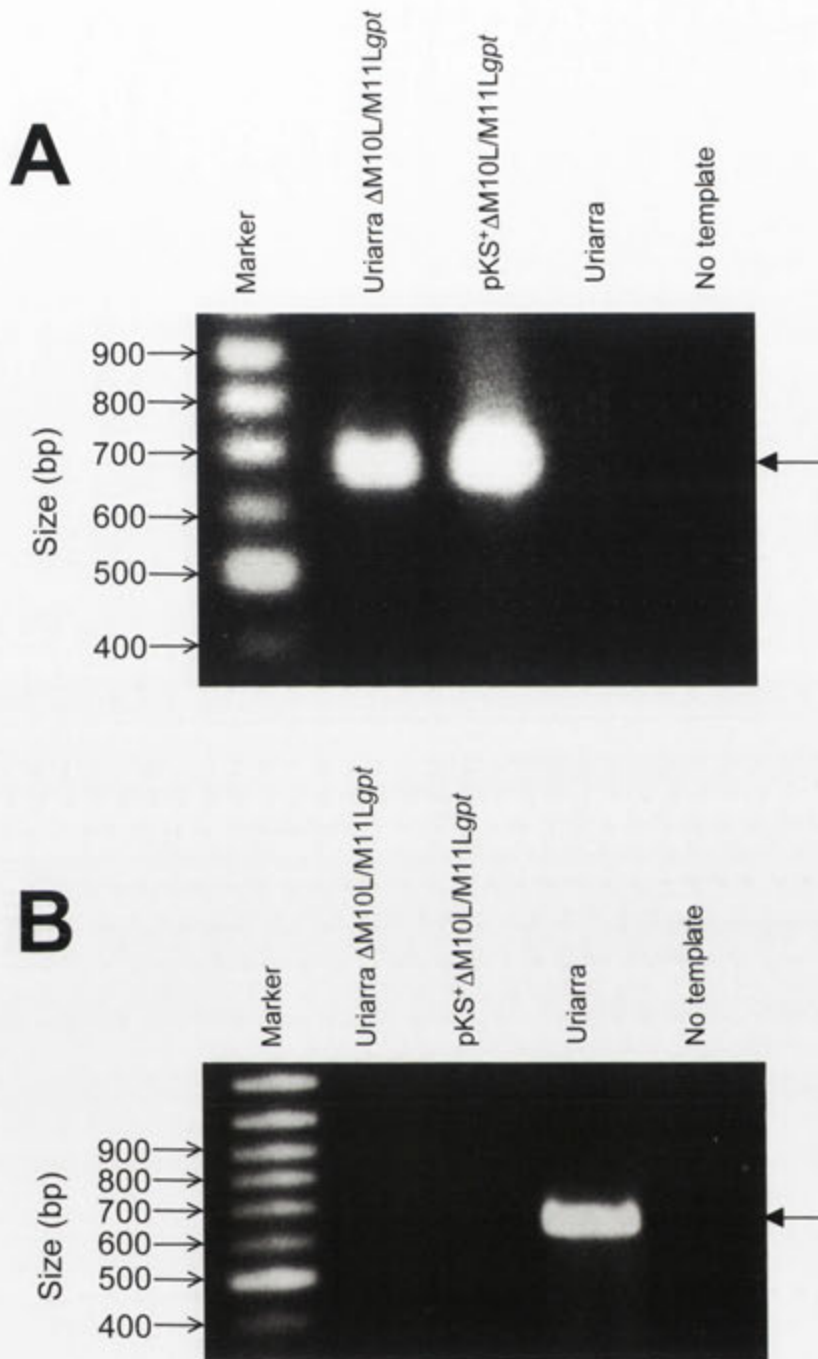


with the p7.5-*gpt* cassette, allowing for selection of recombinant virus by resistance to mycophenolic acid. This is a larger deletion of the open reading frames than that used in the construction of a Lausanne strain with inactivated M010L and M011L ORFs (Opgenorth *et al.*, 1992), where both open reading frames were interrupted by insertion of the *E.coli*  $\beta$ -galactosidase gene at a specific restriction enzyme site in the overlapping section of the two genes.

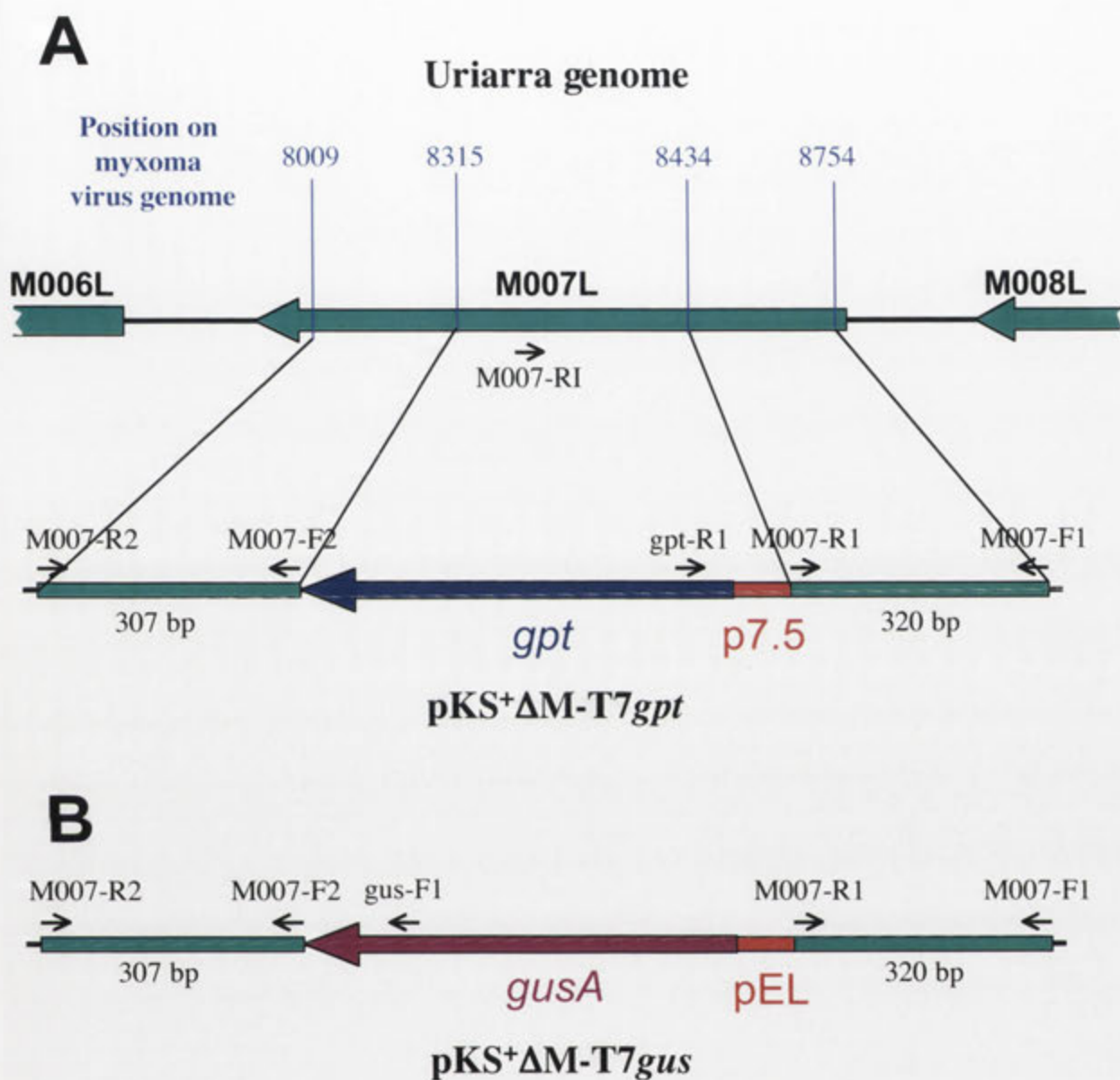
Following infection of RK13 cells with Uriarra and transfection with pKS<sup>+</sup> $\Delta$ M10L/M11L*gpt*, recombinant virus was isolated by plaque purification under mycophenolic acid selection (Section 2.5.3). After 4 rounds of single plaque purification, a pure recombinant virus was obtained and named Uriarra  $\Delta$ M10L/M11L*gpt*. The virus was amplified and concentrated as described in Section 2.5.4. This concentrated virus preparation was found to contain no wild-type virus by PCR analysis (Figure 4.2). Cloning and sequencing of the M010L/M011L locus of Uriarra  $\Delta$ M10L/M11L*gpt* showed the sequence to be the same as the plasmid pKS<sup>+</sup> $\Delta$ M10L/M11L*gpt*, as was expected after a homologous recombination event between this plasmid and Uriarra genomic DNA (data not shown).

#### 4.2.1.2 Design and construction of pKS<sup>+</sup> $\Delta$ M-T7*gpt* and Uriarra $\Delta$ M-T7*gpt*

The inactivation of the two copies of the open reading frame M007 was achieved by homologous recombination in Uriarra-infected RK13 cells with the plasmid pKS<sup>+</sup> $\Delta$ M-T7*gpt* (Figure 4.3A). This plasmid contains two regions of sequence homologous to the 3' and 5' ends of M007, between which was cloned *gpt* under control of the p7.5 promoter. A recombination event between the plasmid and the Uriarra genome would result in a 118 bp



**Figure 4.2 - PCR analysis of Uriarra  $\Delta$ M10L/M11Lgpt.** DNA isolated from the concentrated Uriarra  $\Delta$ M10L/M11Lgpt virus stock was analysed by PCR primer pairs specific for recombinant virus (A; gpt-R1 x M012-F1, 645 bp) or wild type virus (B; M010L-R1 x M012L-F1, 684 bp). The plasmid pKS<sup>+</sup> $\Delta$ M10L/M11Lgpt was used as a positive control for recombinant virus, and Uriarra genomic DNA was used as a positive control for wild type virus. No wild type PCR product was amplified from the Uriarra  $\Delta$ M10L/M11Lgpt virus preparation (B, arrowed).



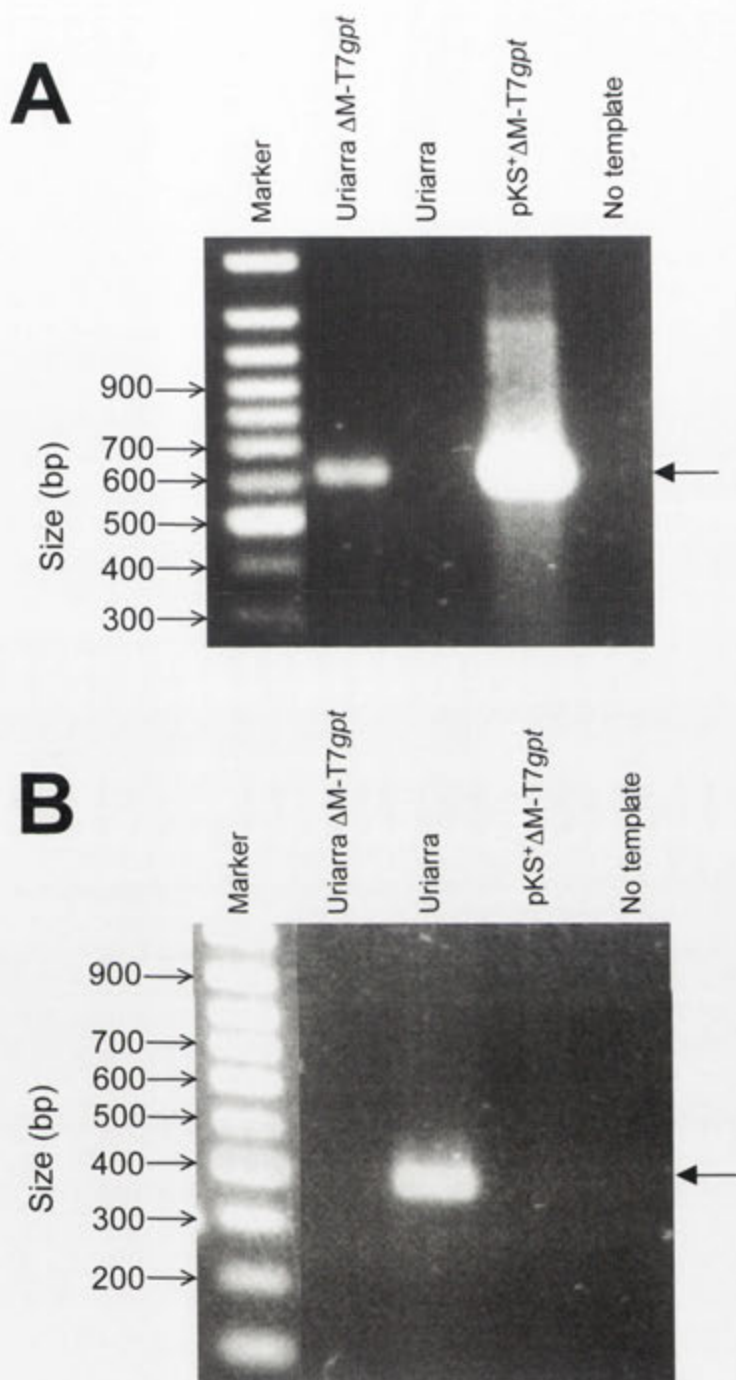
**Figure 4.3 - Construction of plasmids for deletion of M007 sequences of myxoma virus.** **A:** Short sequences (307 and 320 bp) homologous to Uriarra DNA were cloned by PCR using the primers indicated (small black arrows; Table 2.3), between which the selective gene *gpt* under control of the p7.5 promoter was cloned. Homologous recombination results in a recombinant virus (Uriarra ΔM-T7*gpt*) with a 118 bp deletion in the middle of each copy of M007. **B:** In place of the *gpt* gene, the β-glucuronidase (*gusA*) gene under control of the synthetic early/late promoter (pEL) was cloned as the selection marker to construct the recombinant virus Uriarra ΔM-T7*gus*. The positions of other primers used for the PCR screening of recombinant viruses (M007-R1, *gpt*-R1 and *gus*-F1) are indicated. The position of the ends of the homologous sequences are indicated as described in the published myxoma virus sequence, Genbank AF170726. Figure is not to scale.

deletion in the open reading frame of M007 (15.1% of the ORF), and its replacement with the p7.5-*gpt* cassette.

Following infection of RK13 cells with Uriarra and transfection with pKS<sup>+</sup>ΔM-T7*gpt*, recombinant virus was isolated by plaque purification under mycophenolic acid selection (Section 2.5.3). After 7 rounds of single plaque purification, a pure recombinant virus was obtained and named Uriarra ΔM-T7*gpt*. The virus was amplified and concentrated as described in Section 2.5.4, and was found to contain no wild-type virus by PCR analysis (Figure 4.4). Cloning and sequencing of the M007 locus of Uriarra ΔM-T7*gpt* showed the sequence to be the same as the plasmid pKS<sup>+</sup>ΔM-T7*gpt*, as was expected after a homologous recombination of the plasmid sequence into the virus (data not shown).

#### 4.2.1.3 Design and construction of pKS<sup>+</sup>ΔM-T7*gus* and Uriarra ΔM10L/M11L*gpt* ΔM-T7*gus* (TKO)

The construction of Uriarra ΔM10L/M11L*gpt* ΔM-T7*gus* was achieved by homologous recombination in Uriarra ΔM10L/M11L*gpt*-infected RK13 cells with the plasmid pKS<sup>+</sup>ΔM-T7*gus* (Figure 4.3B). This plasmid was the same as pKS<sup>+</sup>ΔM-T7*gpt* except for the replacement of the p7.5-*gpt* cassette with a pEL-*gus* cassette, containing the *E. coli* β-glucuronidase gene (*gusA*) under control of the synthetic early/late poxvirus promoter pEL (Davidson and Moss, 1989). This method, first developed by Carroll and Moss (1995), is routinely used for the construction and selection of recombinant poxviruses. A recombination event between the genome of Uriarra ΔM10L/M11L*gpt* and pKS<sup>+</sup>ΔM-T7*gus* would result in an identical 118 bp deletion in the M007 ORF as found in Uriarra ΔM-T7*gpt*, and its replacement with the pEL-*gus* cassette, allowing for the selection of recombinant virus by identification of plaques which stain blue in the presence of X-gluc.



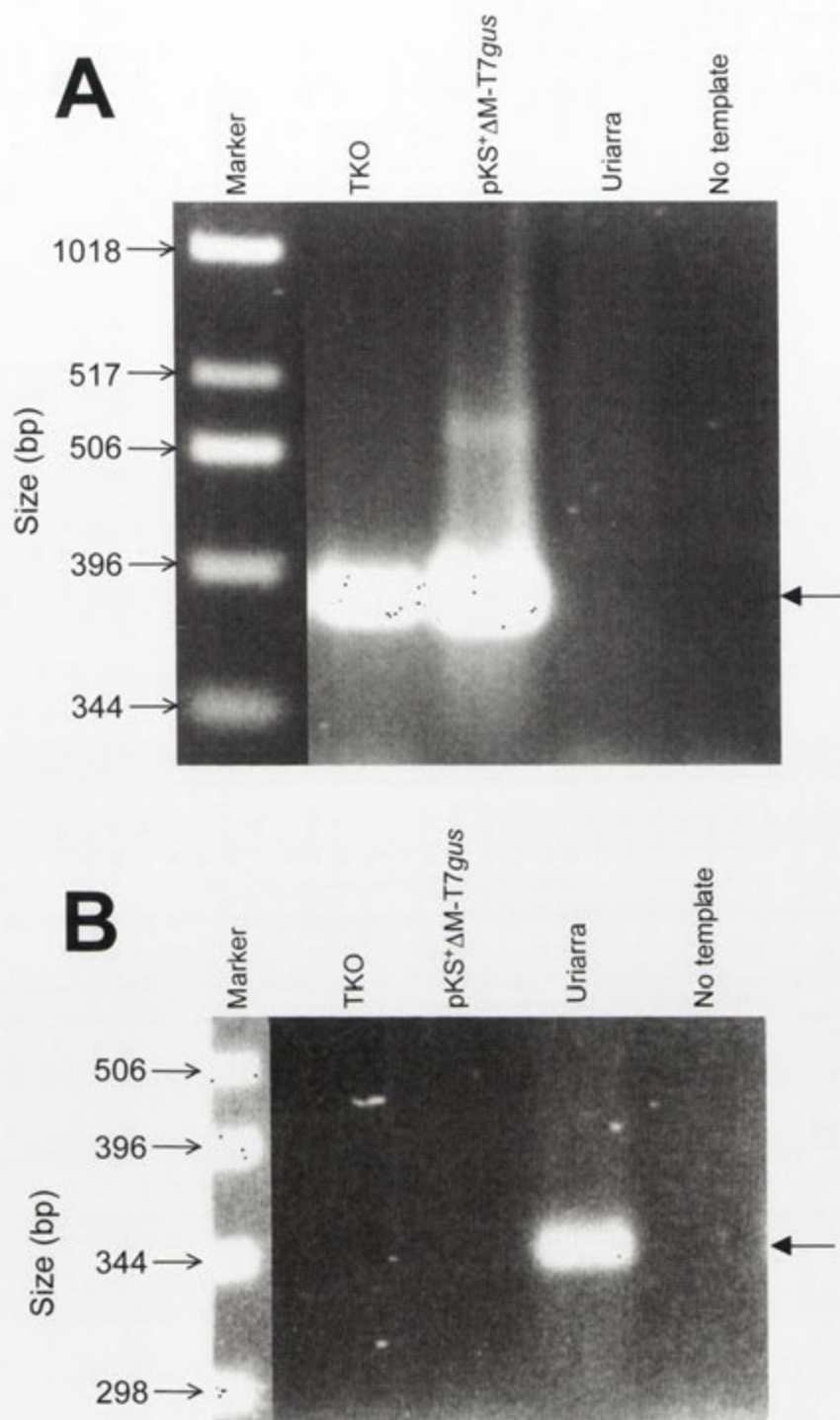
**Figure 4.4** - PCR analysis of Uriarra  $\Delta$ M-T7gpt. DNA isolated from the concentrated Uriarra  $\Delta$ M-T7gpt virus stock was analysed by PCR primer pairs specific for recombinant virus (A; gpt-R1 x M007-F1, 603 bp) or wild type virus (B; M007-RI x M007-F1, 380 bp). The plasmid pKS<sup>+</sup> $\Delta$ M-T7gpt was used as a positive control for recombinant virus, and Uriarra genomic DNA was used as a positive control for wild type virus. No wild type PCR product was amplified from the Uriarra  $\Delta$ M-T7gpt virus preparation (B, arrowed).

Following infection of RK13 cells with Uriarra  $\Delta$ M10L/M11Lgpt and transfection with pKS<sup>+</sup> $\Delta$ M-T7gus, recombinant virus was isolated by single plaque purification after selection of isolated plaques that stained blue in the presence of X-gluc (Section 2.5.3.4). After 8 rounds of single plaque purification, a pure recombinant virus was obtained and named Uriarra  $\Delta$ M10L/M11Lgpt  $\Delta$ M-T7gus, or Triple KnockOut (TKO). The virus was amplified and concentrated as described in Section 2.5.4, and was found to contain no Uriarra or Uriarra  $\Delta$ M10L/M11Lgpt by PCR analysis (Figure 4.5). Cloning and sequencing of the M010L/M011L and M007 loci of TKO showed the sequence to be the same as the plasmids pKS<sup>+</sup> $\Delta$ M10L/M11Lgpt and pKS<sup>+</sup> $\Delta$ M-T7gus respectively, as was expected after a homologous recombination of the plasmid sequences into the virus (data not shown).

#### **4.2.2 *In vitro* analysis of recombinant virus replication and protein secretion characteristics**

Previous studies involving the deletion of M007, M011L and M010L indicated that the deletions had no effect on the *in vitro* growth characteristics of myxoma virus in rabbit and primate fibroblast cells lines (Mossman *et al.*, 1996b; Opgenorth *et al.*, 1992). The attenuation of the recombinant viruses observed *in vivo* was therefore attributed to enhanced host responses to the viruses due to the loss of the viral immunomodulatory genes (Barrett *et al.*, 2001). Several *in vitro* tests were performed to confirm the deletion of the viral sequences in the recombinant Uriarra virus strains, and to investigate the *in vitro* growth characteristics of the recombinant viruses compared to their parental strain, Uriarra.





**Figure 4.5 - PCR analysis of TKO.** DNA isolated from the concentrated TKO virus stock was analysed by PCR primer pairs specific for recombinant virus (A; gus-F1 x M007-R2, 411 bp) or wild type virus (B; M007-RI x M007-F1, 380 bp). The plasmid pKS<sup>+</sup>ΔM-T7gus was used as a positive control for recombinant virus, and Uriarra genomic DNA was used as a positive control for wild type virus. No wild type PCR product was amplified from the TKO virus preparation (B, arrowed).

#### 4.2.2.1 Growth characteristics of recombinant viruses in RK13 cells

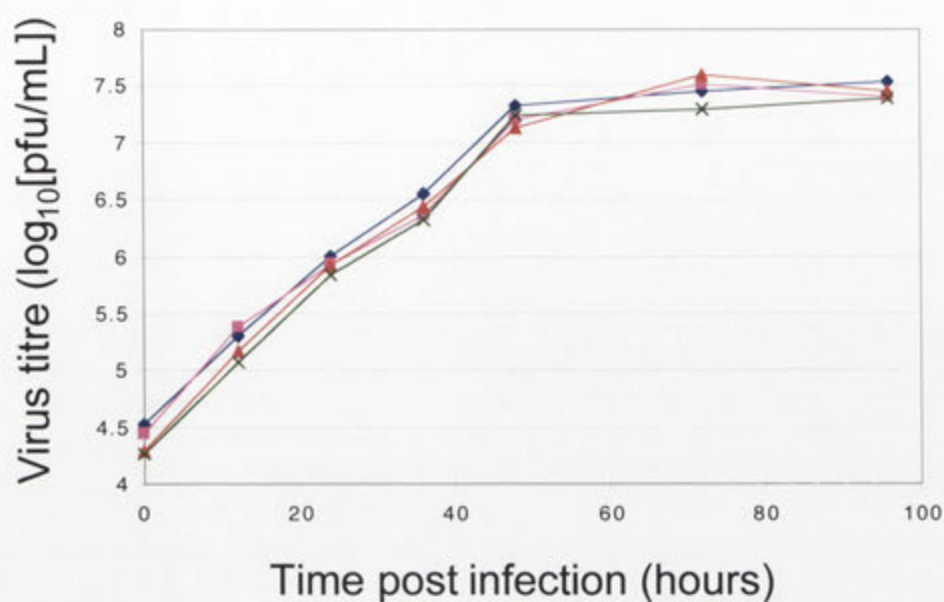
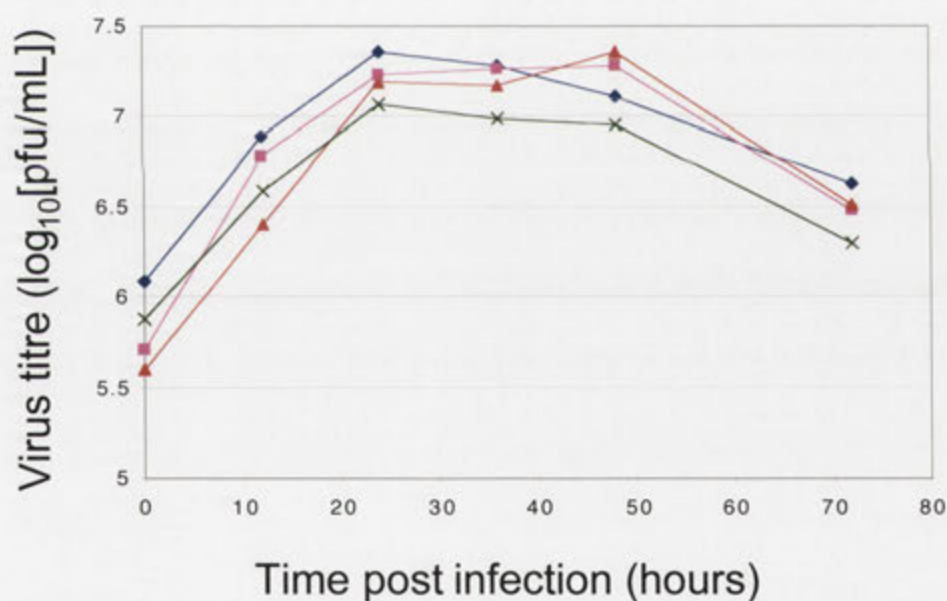
To determine the growth characteristics of Uriarra  $\Delta$ M10L/M11Lgpt, Uriarra  $\Delta$ M-T7gpt and TKO compared to their parental virus Uriarra, RK13 cell monolayers were infected with the viruses at a multiplicity of infection (MOI) of 0.1 or 3.0, and the titre of virus in duplicate samples determined at various time points following infection (Section 2.5.6). Uriarra and the recombinant viruses all had comparable growth characteristics, increasing in titre over time to very similar levels (Figure 4.6). It was concluded that the deletions of M010L, M011L and M007 in Uriarra  $\Delta$ M10L/M11Lgpt, Uriarra  $\Delta$ M-T7gpt and TKO have no effect on virus replication in RK13 cells.

#### 4.2.2.2 Growth of recombinant viruses in RL-5 cells and viability of RL-5 cells following infection with recombinant viruses

The deletion of M011L results in a virus with reduced ability to prevent the apoptosis of infected lymphocytes, both *in vivo* and *in vitro* (Everett *et al.*, 2000; Opgenorth *et al.*, 1992). This resulted in reduced growth of  $\Delta$ M011L viruses in rabbit lymphocytes, and decreased viability of RL-5 cells infected with  $\Delta$ M011L myxoma viruses as observed by trypan blue exclusion assay (Everett *et al.*, 2000). To confirm that the function of M011L had been interrupted in Uriarra  $\Delta$ M10L/M11Lgpt and TKO, the replication of each virus in the rabbit lymphocyte cell line RL-5, and the effect of virus infection on RL-5 viability was measured.

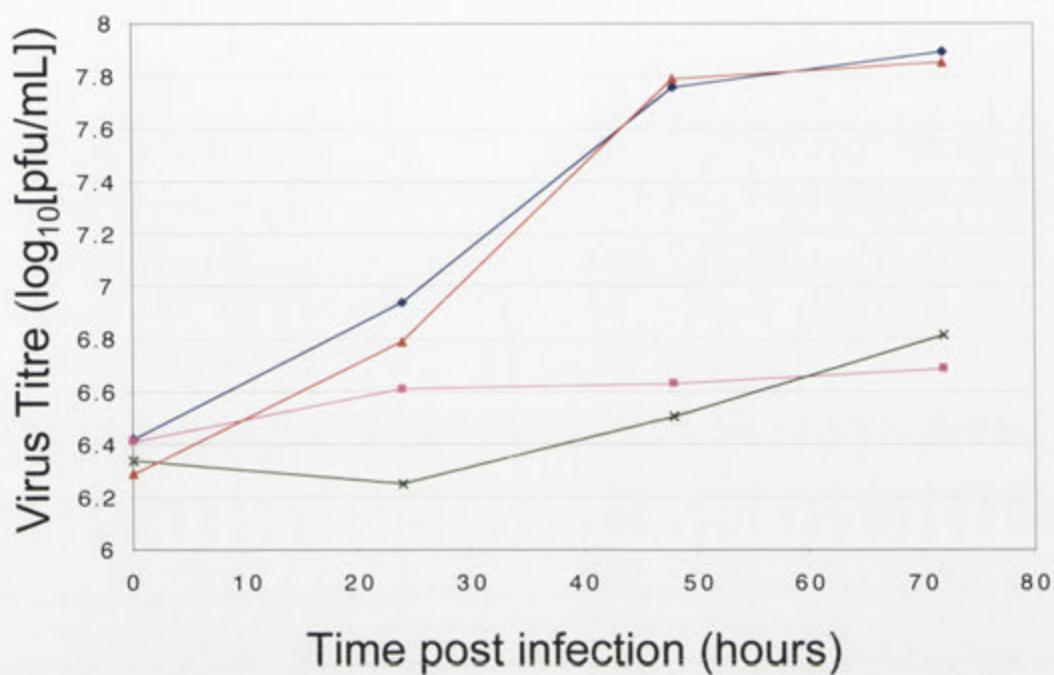
RL-5 cells were infected with Uriarra, Uriarra  $\Delta$ M10L/M11Lgpt, Uriarra  $\Delta$ M-T7gpt or TKO at a MOI of 3.0 and the titre of virus determined at 24, 48 or 72 hours after infection (Section 2.5.7). Both Uriarra and Uriarra  $\Delta$ M-T7gpt replicated in RL-5 cells, with little difference between the two strains, increasing from approximately  $2.5 \times 10^6$  pfu/mL to



**A****B**

**Figure 4.6 - Growth of recombinant viruses in RK13 cells.** RK13 cells were infected with virus at an MOI of 0.1 (A) or 3.0 (B) and the titre of virus following infection determined by plaque assay. Each point represents the mean of duplicate samples.

Key: Uriarra - ◆; Uriarra  $\Delta$ M10L/M11Lgpt - ■; Uriarra  $\Delta$ M-T7gpt - ▲; TKO - ×.



**Figure 4.7 - Growth curve of vaccine candidates and Uriarra in RL-5 cells.** RL-5 cells were infected with recombinant viruses at an MOI of 3 and the titre of virus in duplicate samples determined every 24 hours post infection.

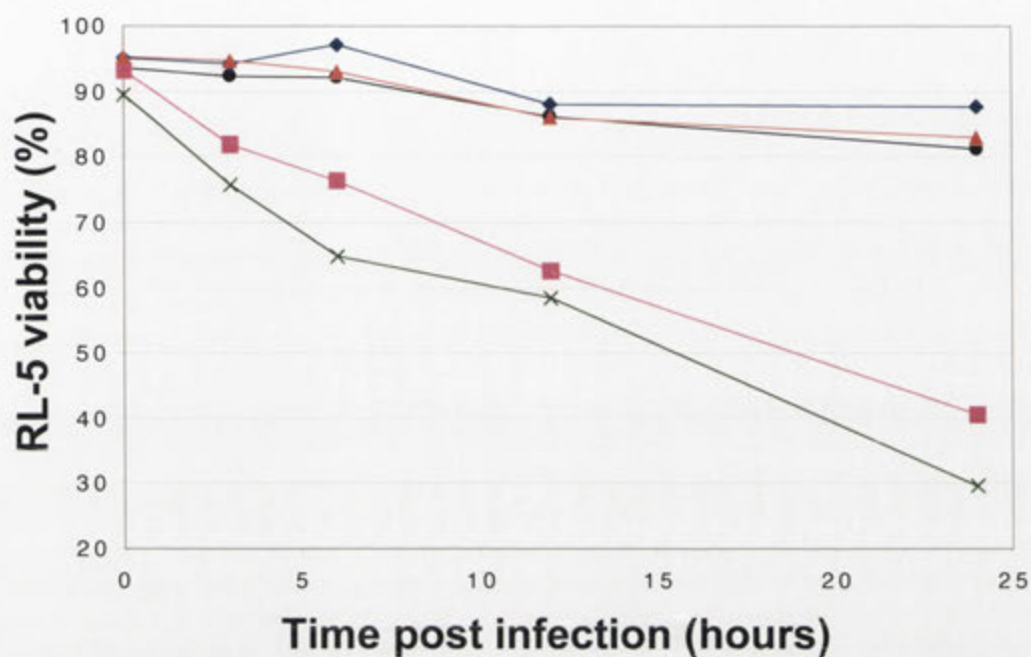
Key: Uriarra - ◆; Uriarra  $\Delta$ M10L/M11Lgpt - ■; Uriarra  $\Delta$ M-T7gpt - ▲; TKO - ✕.

approximately  $8 \times 10^7$  pfu/mL by 72 hours post infection (Figure 4.7). However, Uriarra  $\Delta M10L/M11Lgpt$  and TKO only increased in titre slightly to approximately  $5 \times 10^6$  pfu/mL, more than one log less than for Uriarra and Uriarra  $\Delta M-T7gpt$ .

The viability of RL-5 cells following infection at an MOI of 10 with Uriarra, Uriarra  $\Delta M10L/M11Lgpt$ , Uriarra  $\Delta M-T7gpt$  or TKO was determined by trypan blue exclusion assay (Section 2.5.8). The viability of Uriarra- and Uriarra  $\Delta M-T7gpt$ -infected RL-5 cells remained high throughout the experiment, dropping to 87.6% and 82.4% respectively at 24 hours after infection, similar to mock-infected cells at 81.3% (Figure 4.8). The viability of Uriarra  $\Delta M10L/M11Lgpt$ - and TKO-infected RL-5 cells dropped rapidly following infection to 40.6 and 29.7% respectively at 24 hours after infection. This indicated that the viability of RL-5 cells infected with  $\Delta M011L$  virus strains was markedly reduced compared to viruses with an intact M011L gene, and compares well with the results of Everett *et al.* (2000). These results, together with the replication of the recombinant viruses in RL-5 cells indicate that Uriarra  $\Delta M10L/M11Lgpt$  and TKO do not possess an active M011L gene.

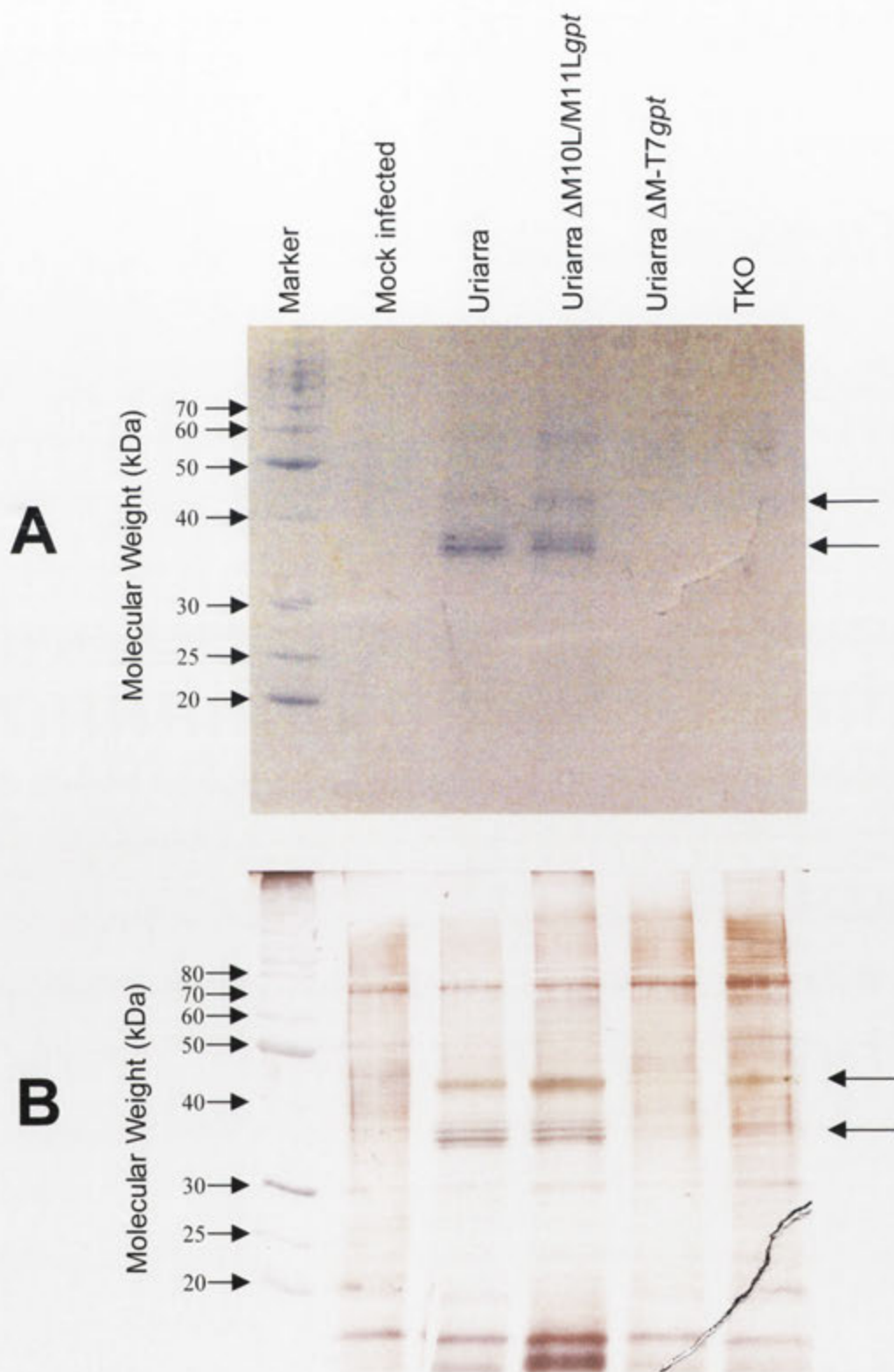
#### 4.2.2.3 Confirmation of deletion of M-T7 sequence in Uriarra $\Delta M-T7gpt$ and TKO

The product of the M007 gene, the IFN- $\gamma$ -binding protein (M-T7), is the major protein secreted from cells infected with myxoma virus (Upton *et al.*, 1992). To confirm that M-T7 protein was not produced in Uriarra  $\Delta M-T7gpt$ - and TKO-infected cells, RK13 cell monolayers were mock infected or infected with Uriarra, Uriarra  $\Delta M10L/M11Lgpt$ , Uriarra  $\Delta M-T7gpt$  or TKO at an MOI of 1, and incubated in serum free media for 24 hours. The media was then concentrated as described in Section 2.4.7 and proteins separated by SDS-PAGE. Figure 4.9 shows the supernatants from virus-infected and mock-infected cells on Coomassie Blue-stained and silver-stained gels. Distinct groups of bands of 37 and 42 kDa



**Figure 4.8** - Viability of RL-5 cells following infection with recombinant myxoma virus strains. RL-5 cells were infected with virus at an MOI of 10 and the viability of duplicate samples determined by trypan blue exclusion.

Key: Mock infected - ●; Uriarra - ◆; Uriarra  $\Delta$ M10L/M11Lgpt - ■; Uriarra  $\Delta$ M-T7gpt - ▲; TKO - ✕.



**Figure 4.9 - Analysis of supernatants from virus infected cells.** After 24 hours incubation, the supernatants from RK13 cells infected with Uriarra, Uriarra  $\Delta$ M10L/M11Lgpt, Uriarra  $\Delta$ M-T7gpt or TKO were separated by SDS-PAGE, and stained with Coomassie Blue (A) or silver stained (B). In both gels, bands at 37 and 42 kDa (arrowed) can be seen in Uriarra and Uriarra  $\Delta$ M10L/M11Lgpt supernatants. Benchmark protein markers were fractionated as size markers.

can be seen in the supernatants of Uriarra- and Uriarra  $\Delta M10L/M11Lgpt$ -infected cells which are absent in the supernatants of cells infected with Uriarra  $\Delta M-T7gpt$  and TKO. The different sizes of the bands possibly indicate the M-T7 gene product is secreted with different levels of post-translational modification, resulting in slightly different molecular weights. These bands correspond with the size of the  $\gamma$ -interferon binding protein observed by Mossman *et al.* (1996). This evidence suggests that Uriarra  $\Delta M-T7gpt$ - and TKO-infected cells do not secrete the M-T7 protein.

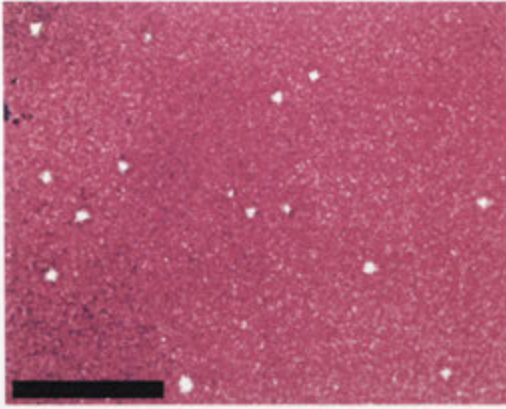
#### 4.2.2.4 Analysis of Uriarra $\Delta M10L/M11Lgpt$ plaque morphology *in vitro*

During the analysis of Uriarra  $\Delta M10L/M11Lgpt$  growth kinetics *in vitro*, it was discovered that this knockout virus had unusual plaque morphology when grown on Vero cell monolayers. Infected cells grown in plaque assays were usually grown for 6 days, and then fixed and stained with crystal violet. After 6 days of growth, the visual appearance of Uriarra and Uriarra  $\Delta M10L/M11Lgpt$  plaques were identical (data not shown). However, after 8 days of growth, a difference was observed (Figure 4.10). The plaques formed by Uriarra  $\Delta M10L/M11Lgpt$  were approximately twice the diameter of Uriarra plaques and exhibited a ‘comet plaque’ morphology. The large primary plaques were usually at the head of a series of smaller secondary plaques that formed in parallel lines ‘behind’ the primary plaques. This plaque morphology is typical of a poxvirus which is producing higher amounts of extracellular enveloped virus (EEV) particles (Smith *et al.*, 2002).

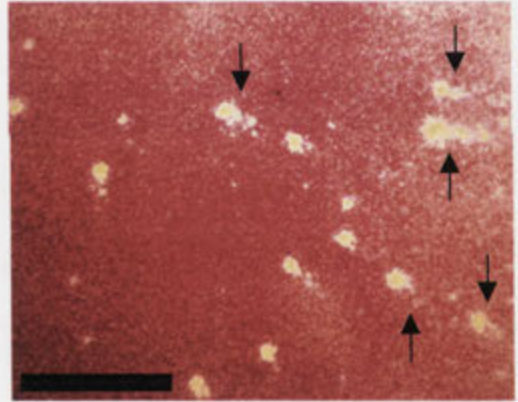
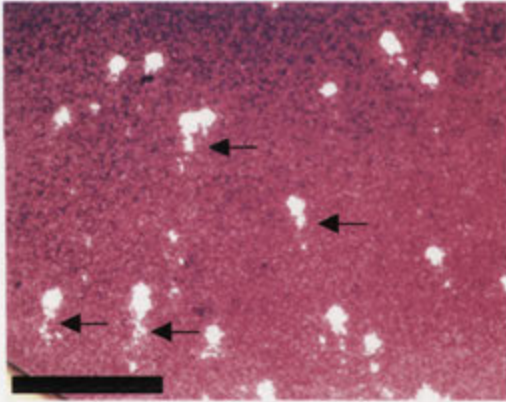
In case the observed plaque phenotype of Uriarra  $\Delta M10L/M11Lgpt$  was caused by an unrelated mutation in another gene during plaque purification, the virus was re-purified from the original recombinant virus preparation (the original Uriarra/pKS<sup>+</sup> $\Delta M10L/M11Lgpt$ -infection/transfection lysate). Six different plaques were



### Uriarra



### Uriarra $\Delta$ M10L/M11Lgpt



**Figure 4.10** - Plaque morphology of Uriarra  $\Delta$ M10L/M11Lgpt on Vero cells. Eight days after infection of monolayers, Uriarra  $\Delta$ M10L/M11Lgpt showed a different plaque morphology to its parental virus (Uriarra) with larger primary plaque sizes and a distinct 'comet plaque' morphology (arrowed). Bars on the images are 5 mm in length.

picked from this preparation and purified. All six had the same plaque phenotype as the original isolate of Uriarra  $\Delta M10L/M11Lgpt$ , suggesting this phenotype is a characteristic of the deletion in the M010L/M011L locus of Uriarra (data not shown). The implications of this plaque phenotype to the vaccine's pathogenesis will be discussed in Section 4.3.3.

### **4.2.3 Vaccination of rabbits with recombinant myxoma virus vaccines**

#### *4.2.3.1 Vaccination of rabbits with Uriarra $\Delta M10L/M11Lgpt$*

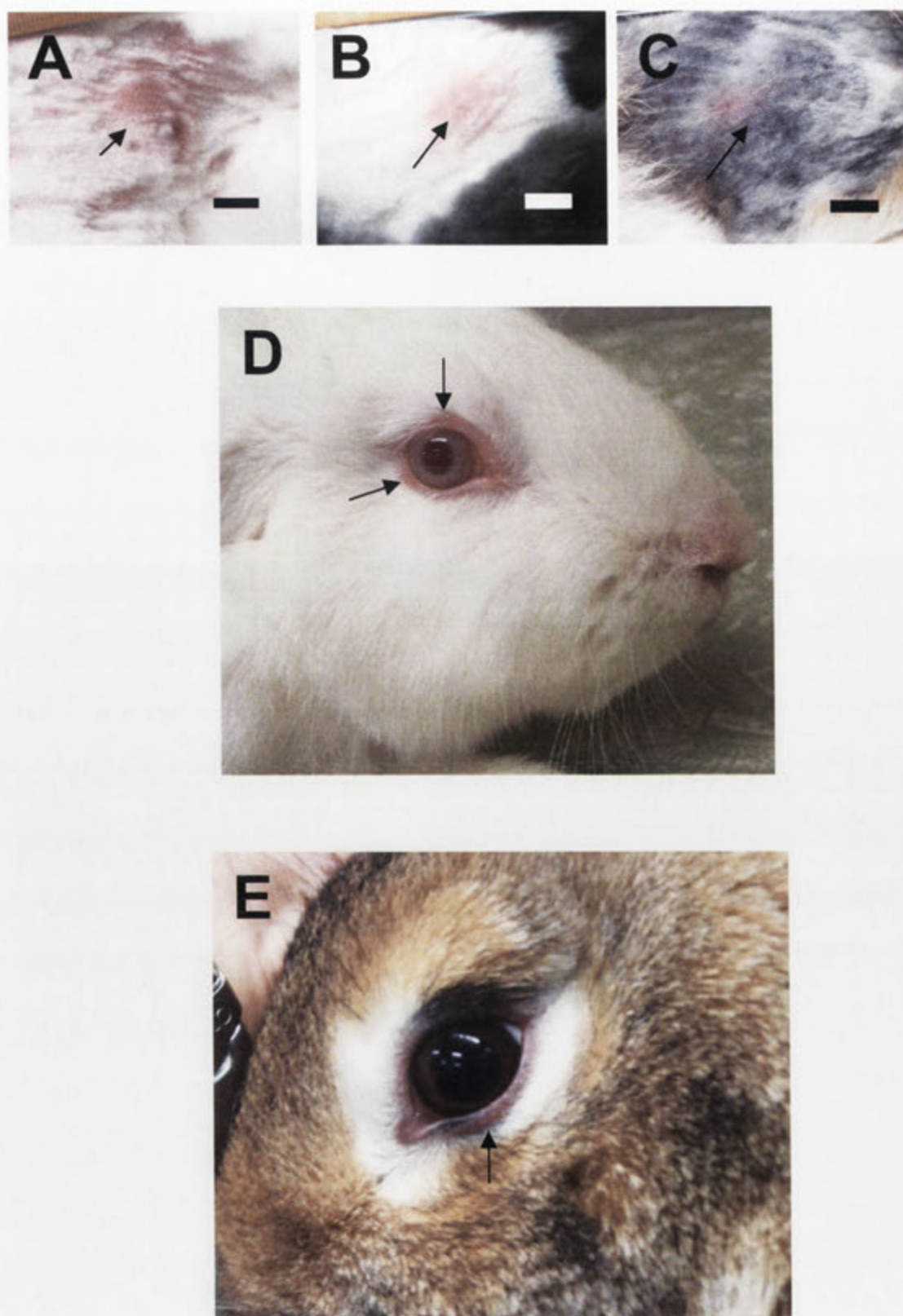
Six male domestic rabbits of between twelve and fourteen months of age were inoculated with 5000 pfu of Uriarra  $\Delta M10L/M11Lgpt$  subcutaneously on the left thigh and examined daily for 3 weeks. Their clinical symptoms are summarised in Table 4.1.

At 3 days post inoculation (dpi), a slight reddening of the skin was seen at the inoculation site, which was 2-5 mm in diameter. By 7 dpi, the inoculation site had developed into a distinct lesion 10-30 mm in diameter (Figure 4.11A-C), and all animals had begun to develop secondary lesions on the eyelids (Figure 4.11D and E). Secondary lesions became more evident on later days, especially on the eyelids, anogenital region, face and ears on all animals. These lesions were red in colour and no larger than 10 mm in diameter (Figure 4.12C-F). The primary lesion ceased growing (a maximum of 20-35 mm in diameter) and became circumscribed by 11 to 13 dpi (Figure 4.12A and B). By 15 dpi, the lesion changed colour from crimson to black and developed a hard brown or black scab on its surface. The lesion then began to regress slightly, changing from crimson to pink at the edges. By 21 dpi, vaccinated animals had recovered from the vaccine virus infection. The primary lesion was scabbed over, or the scab had come off (Figure 4.13A-C). Secondary lesions were still evident on many animals, although they had all scabbed over (Figure 4.13D and F) or regressed completely (Figure 4.13E). Thirty weeks following vaccination, scarring was

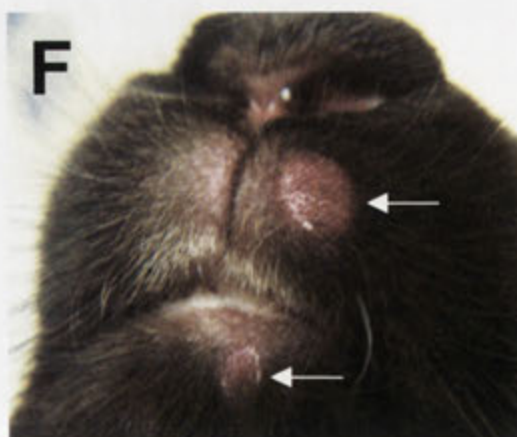
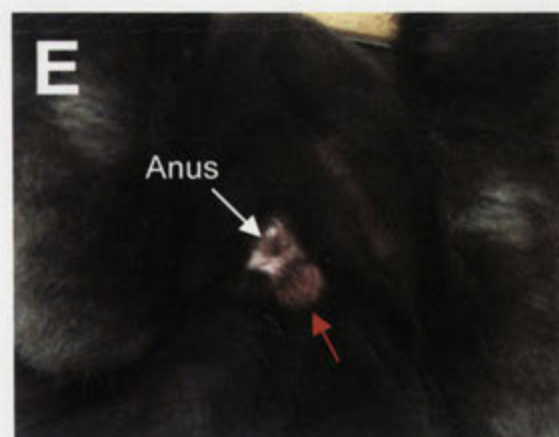
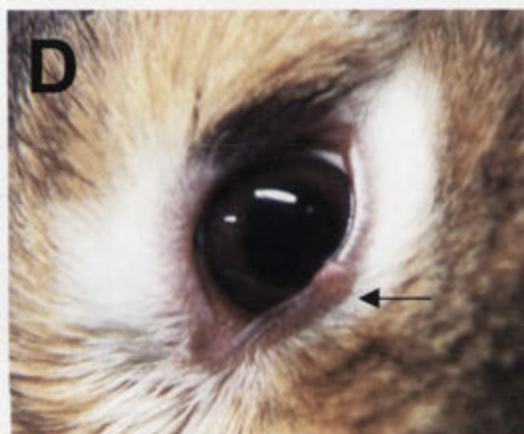
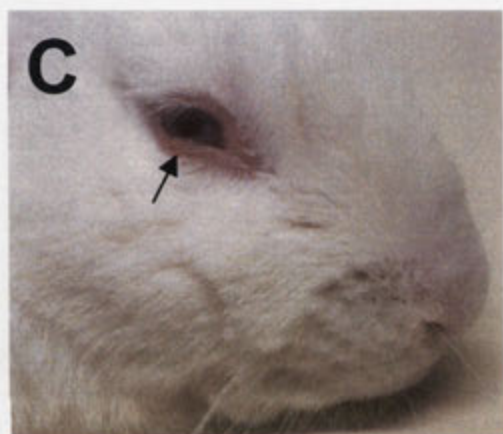
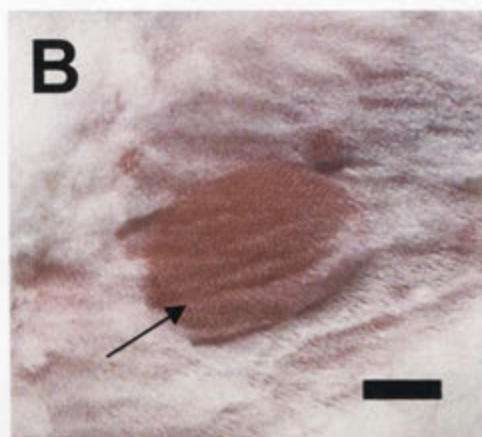
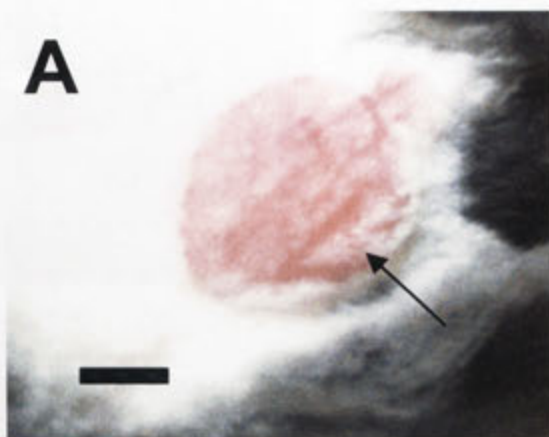


**Table 4.1** – Clinical signs of mature domestic rabbits following vaccination with 5000 pfu of Uriarra ΔM10L/M11Lgpt.

Day post inoculation	Clinical signs
3	Slight reddening of skin at the inoculation site
6	Primary lesion 10-20 mm in diameter. Some animals had red areas on the eyelids.
7	Primary lesion 10-30 mm in diameter. Secondary lesions evident on eyelids of most animals.
8	Primary lesion 15-28 mm in diameter. Temperatures above 40°C. Secondary lesions on eyes approximately 4 mm in diameter, red in colour. One animal had very mild anogenital swelling.
10	Primary lesion 20-35 mm in diameter, some showing black haemorrhagic patches. 2/6 rabbits have temperatures above 40°C. Secondary lesions evident on face, ears, body or anus on all animals.
15	Primary lesion covered with black scab, circumscribed. Secondary lesions on eyes, face, ears, body scabbing over, regressing. Two animals (019 and 963) had a rash of small (5 mm) secondary lesions over the body.
21	Primary lesion scab has come off. Animals fully recovered from infection although some scabbing and evidence of scarring at sites of secondary lesions.

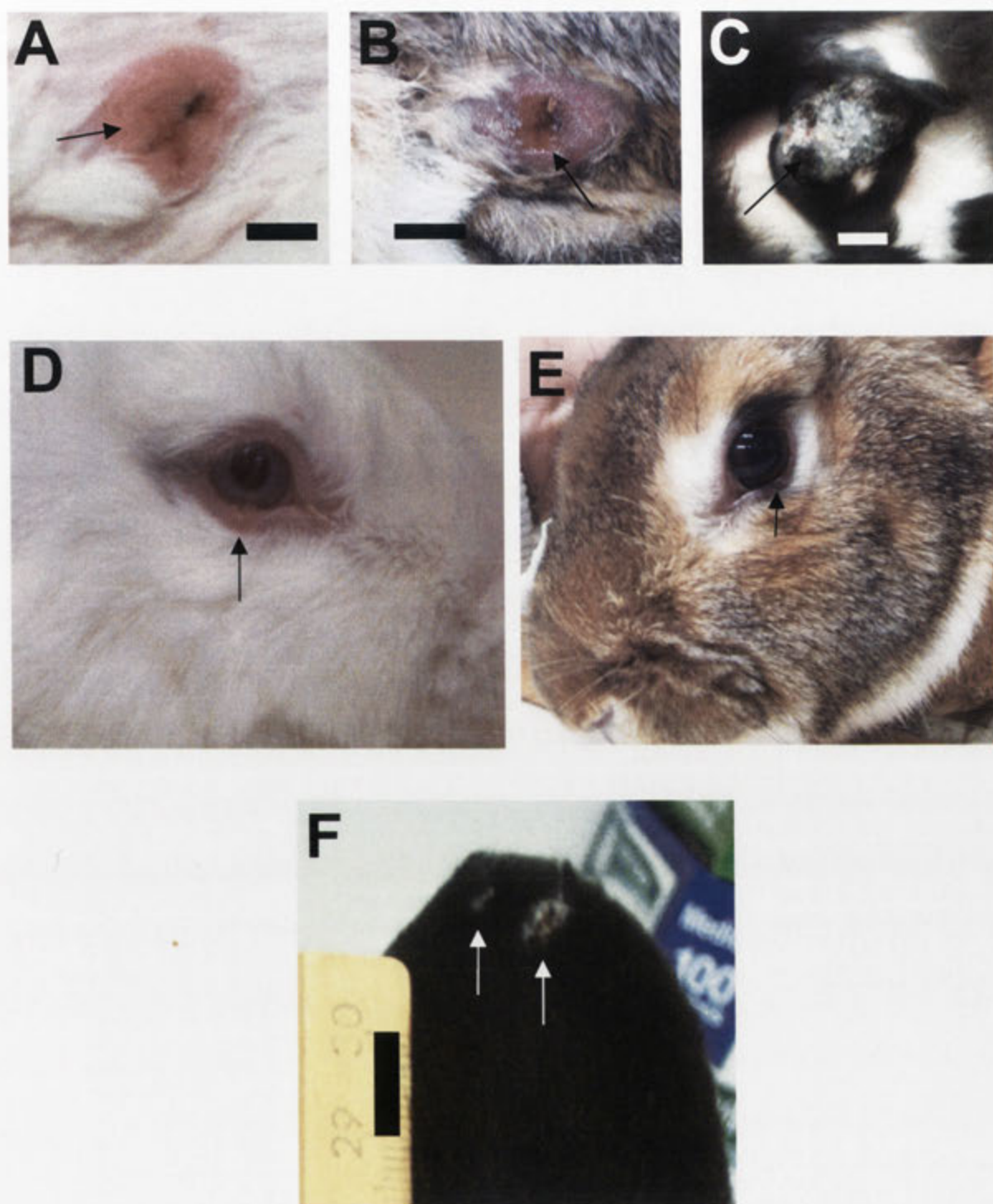


**Figure 4.11** - Rabbits 7 days following inoculation with 5000 pfu of *Uriarra*  $\Delta$ M10L/M11Lgpt. Primary lesions were 10-30 mm raised pink or red lumps (A-C, arrowed). Red patches on the eyelids (C and D; arrowed). Bars on images are 10 mm in size.



**Figure 4.12** - Rabbits 13 days following inoculation with 5000 pfu of Uriarra  $\Delta$ M10L/M11Lgpt. Primary lesions (A and B, arrowed). Secondary lesions at distal sites such as the eyelids (C and D, arrowed), the anus (E, red arrow) or face (F, arrowed). Bars on images are 10 mm in size.





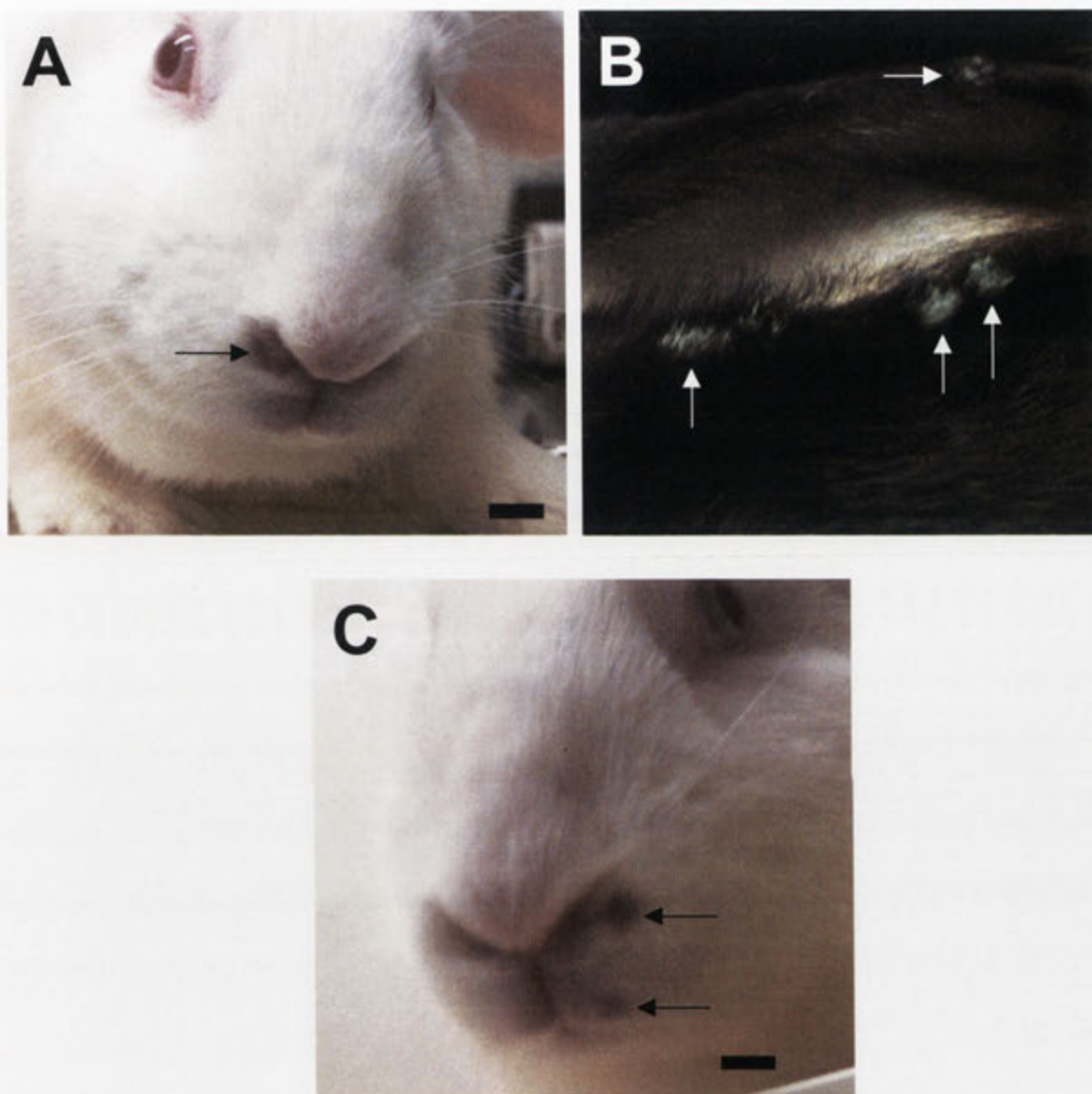
**Figure 4.13** - Rabbits 21 days after inoculation with 5000 pfu of Uriarra  $\Delta$ M10L/M11Lgpt. Primary lesions (A-C, arrowed). Secondary lesions on the eyes (D and E, arrowed) or ears (F, arrowed) had scabbed over or regressed completely (E, arrowed). Bars on images are 10 mm in size.

evident at the sites of secondary lesions on most animals. This scarring was evident as patches of lost fur or fur regrowth of a different colour (Figure 4.14).

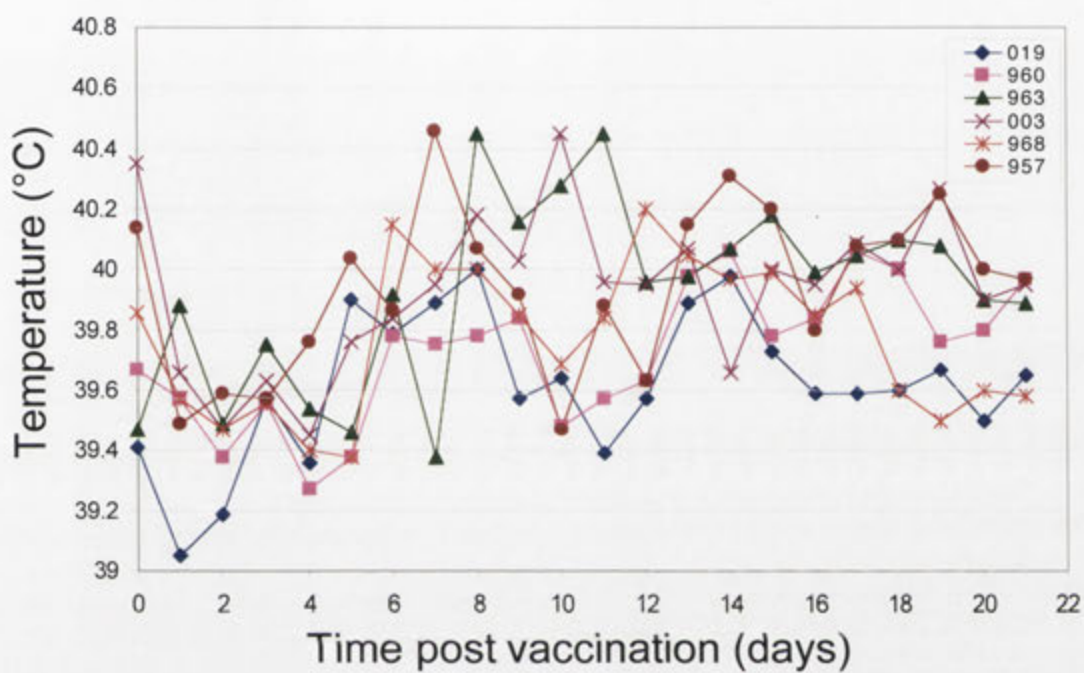
Temperatures were recorded daily for each animal and are shown in Figure 4.15. Temperatures of some animals increased to over 40°C from 5 dpi and at least one animal had a fever every day until 20 dpi. During the 3 weeks of monitoring, all six rabbits had temperatures of 40°C or above for at least 2 days from 5 dpi. Rabbits 957 and 963 had temperatures of greater than 40°C on 10 days between 5 and 20 dpi.

#### *4.2.3.2 Analysis of virus isolated from secondary lesions following vaccination with Uriarra $\Delta$ M10L/M11Lgpt*

Thirteen days following vaccination, biopsies from the primary lesions and secondary lesions of Uriarra  $\Delta$ M10L/M11Lgpt-infected rabbits were collected and used to infect RK13 cells *in vitro*. Virus was grown without mycophenolic acid selection to permit the replication of both recombinant and wild type viruses. From these cells, viral DNA was isolated and examined by PCR to identify wild type and recombinant virus. Uriarra  $\Delta$ M10L/M11Lgpt-specific PCR product was isolated from most sites, including secondary sites as indicated by the 645 bp M012L-F1 x gpt-R1 product amplified from viral DNA (Figure 4.16A). A Uriarra-specific, 599 bp M012L-F1 x M010L-R1 PCR product was not amplified from any viral DNA sample, except the Uriarra DNA control (Figure 4.16B). These results indicate that secondary lesion formation was the result of dissemination of Uriarra  $\Delta$ M10L/M11Lgpt, and not due to a low-level contamination of wild-type Uriarra.

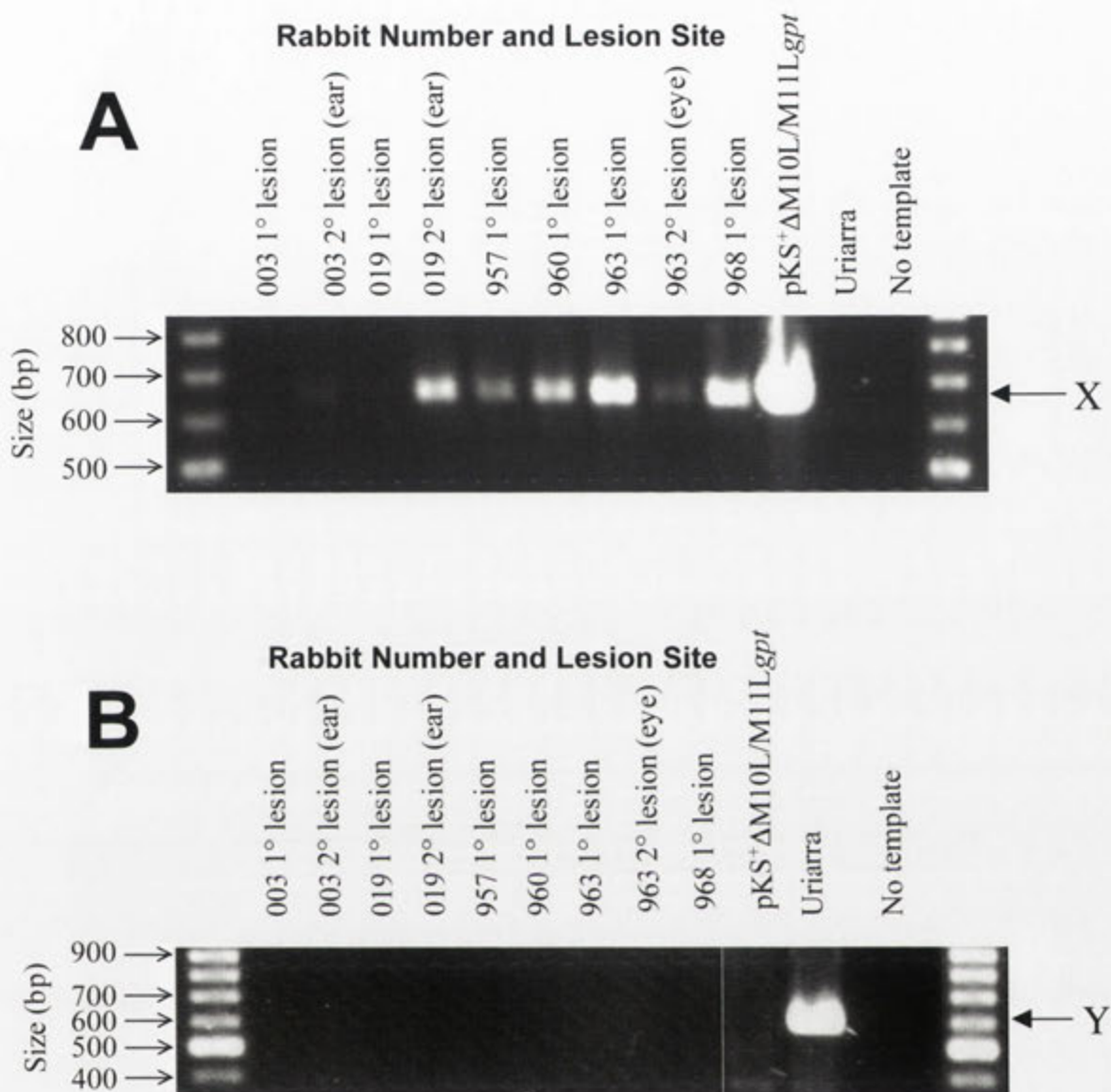


**Figure 4.14** - Scarring on rabbits inoculated with Uriarra  $\Delta$ M10L/M11Lgpt 30 weeks after vaccination. Scarring was evident where secondary lesions developed following inoculation with the vaccine virus (arrowed). Bars on images are 10 mm in size.



**Figure 4.15** - Rectal temperatures of rabbits following inoculation with 5000 pfu of Uriarra  $\Delta$ M10L/M11Lgpt.





**Figure 4.16** - PCR analysis of virus isolated from primary and secondary lesions of Uriarra ΔM10L/M11Lgpt-vaccinated rabbits. DNA was isolated from virus-infected cells seeded with tissue from vaccinated rabbits (rabbits 003, 019, 957, 960, 963 and 968) collected 13 days after vaccination and analysed by PCR to identify recombinant virus (A) or wild type virus (B). The plasmid pKS<sup>+</sup>ΔM10L/M11Lgpt was used as a positive control for recombinant virus, and Uriarra genomic DNA was used as a positive control for wild type virus. A recombinant virus PCR product was amplified from most samples, including tissue collected from secondary lesions (panel A, 645 bp, arrow X). No wild type PCR product was amplified from any site (panel B, 684 bp, arrow Y).



#### 4.2.3.3 Vaccination of rabbits with Uriarra $\Delta M$ -T7gpt

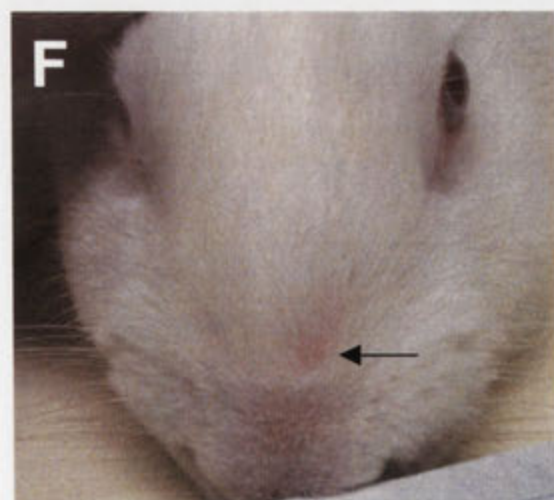
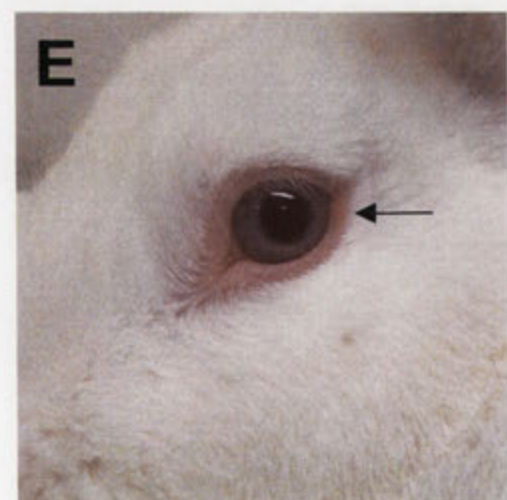
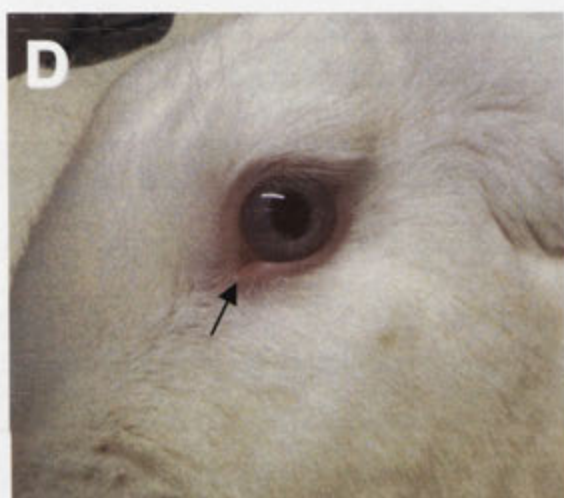
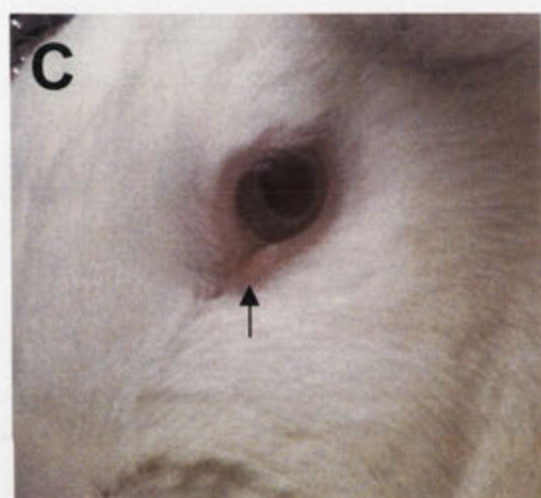
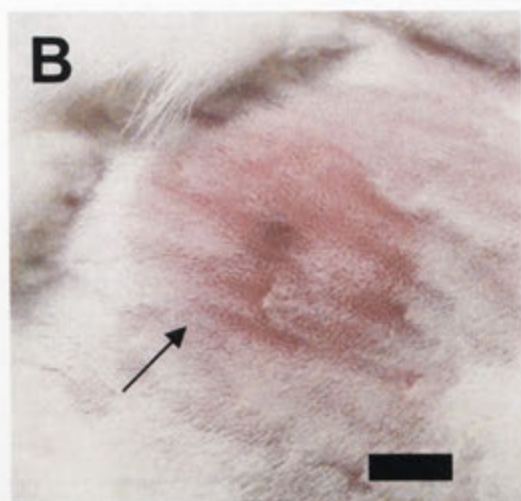
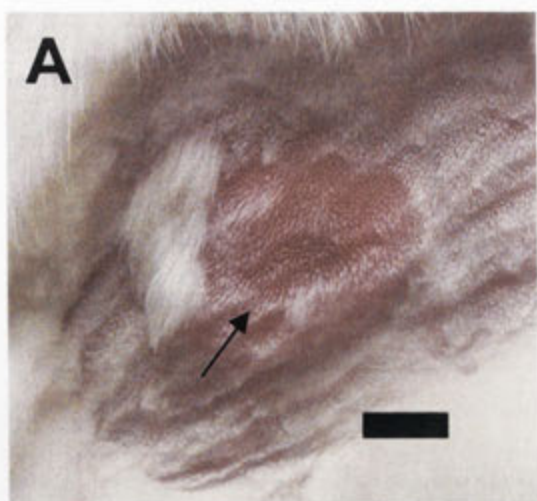
Six domestic male rabbits of between twelve and nineteen months of age were inoculated with 5000 pfu of Uriarra  $\Delta M$ -T7gpt and examined for 15 days. A summary of the clinical signs of these animals is displayed in Table 4.2.

Within 2-3 dpi, a reddening of the skin was seen at the inoculation site, which developed into a primary lesion of 10-25 mm diameter. By 6 dpi, this lesion had grown to 25-35 mm diameter and was pink to red in colour and protuberant (Figure 4.17A and B). In addition, red areas were seen on the eyelids of all animals except rabbit 943 and by 6 dpi, these had developed into secondary lesions (Figure 4.17C-E). Lesions had begun to develop at other sites including the ears, anus and face (Figure 4.17F). At 8 dpi, the primary lesions were 35-50mm in diameter, red in colour, protuberant and some had darker patches in the middle (Figure 4.18A-B). Secondary lesions were more evident on the eyelids, face and ears (Figure 4.18C-F) of all animals except rabbit 943. By 10 dpi, the primary lesion was circumscribed, crimson in colour and many had haemorrhagic patches.

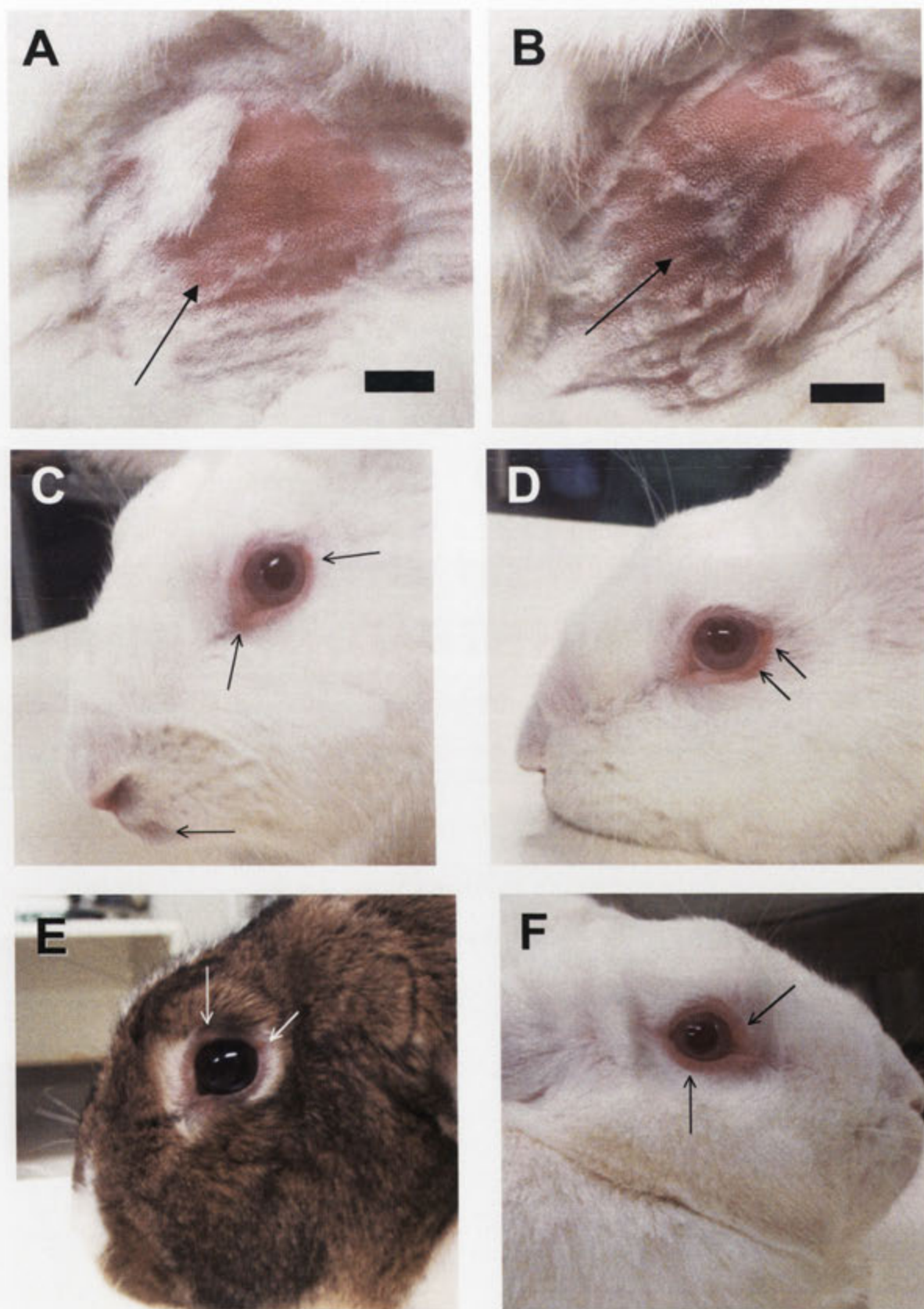
By 15 dpi, the primary lesions were covered in a hard black scab (Figure 4.19A and B) and all animals except rabbit 030 had recovered from infection. Secondary lesions had scabbed over or had regressed on most animals (Figure 4.19C-D). Rabbit 030 had a more severe and persistent infection, had lost weight and had a slightly depressed demeanour. Lesions were more numerous on this animal, especially on the face and eyelids (Figure 4.19E-F). Very mild mucopurulent discharge was observed from the right eye of this animal (Figure 4.19F). Rabbit 030 recovered completely from infection by 21 dpi.

**Table 4.2** – Clinical signs of mature domestic rabbits following vaccination with 5000 pfu of Uriarra  $\Delta$ MT-7gpt.

Day post inoculation	Clinical signs
2	Slight reddening of skin at the inoculation site
3	Primary lesions were pink in colour, 10-25 mm in diameter.
5	Primary lesion 25-35 mm in diameter, pink, raised and firm. Eyelids of 5/6 animals had developed reddening or distinct lesions. Two animals had temperatures above 40°C.
8	Primary lesions 35-50 mm in diameter, raised, red. Secondary lesions (< 8 mm diameter) on 5/6 animals on ears, anus, eyelids and face.
10	Primary lesions circumscribed, 35-50 mm diameter, crimson in colour with black centre. Secondary lesions circumscribed, red, some beginning to scab over. 3/6 animals with temperatures above 40°C.
15	Primary lesions covered in hard black scab. Secondary lesions regressing or scabbed over. One rabbit (030) had not fully recovered and had lost some weight.
21	Primary lesion scab had come off. Animals fully recovered from infection although evidence of scarring at sites of secondary lesions.

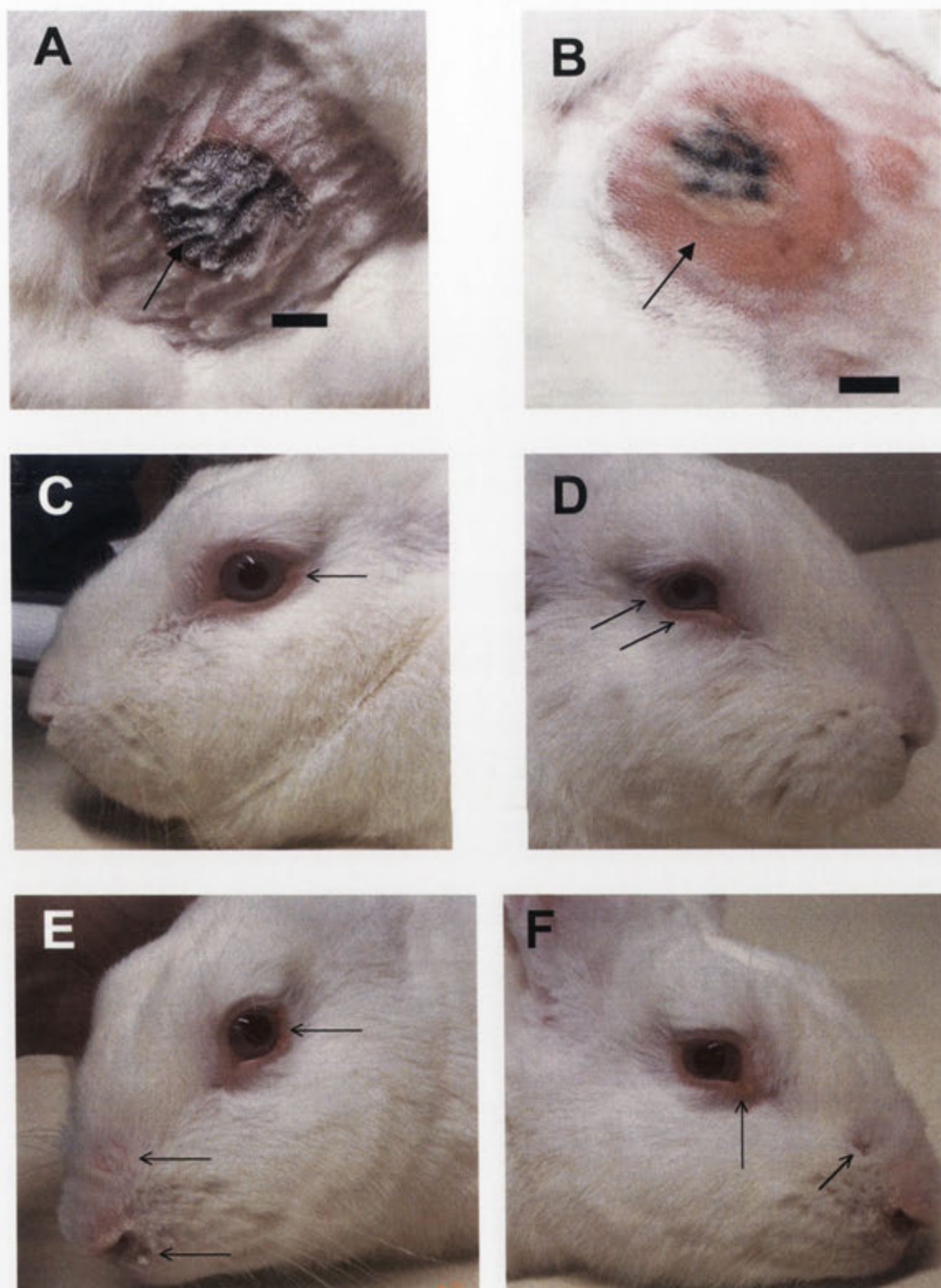


**Figure 4.17** - Rabbits 6 days after inoculation with 5000 pfu of Uriarra  $\Delta M-T7gpt$ . Primary lesions were at least 30-40 mm in diameter, red and protuberant (A and B, arrowed). Secondary lesions on the eyelids (C-E, arrowed) and face (F, arrowed). Bars on images are 10 mm in size.



**Figure 4.18** - Rabbits 8 days after inoculation with 5000 pfu of Uriarra  $\Delta$ M-T7gpt. Primary lesions (A and B, arrowed). Secondary lesions on the eyelids and face (C-F, arrowed). Bars on images are 10 mm in size.





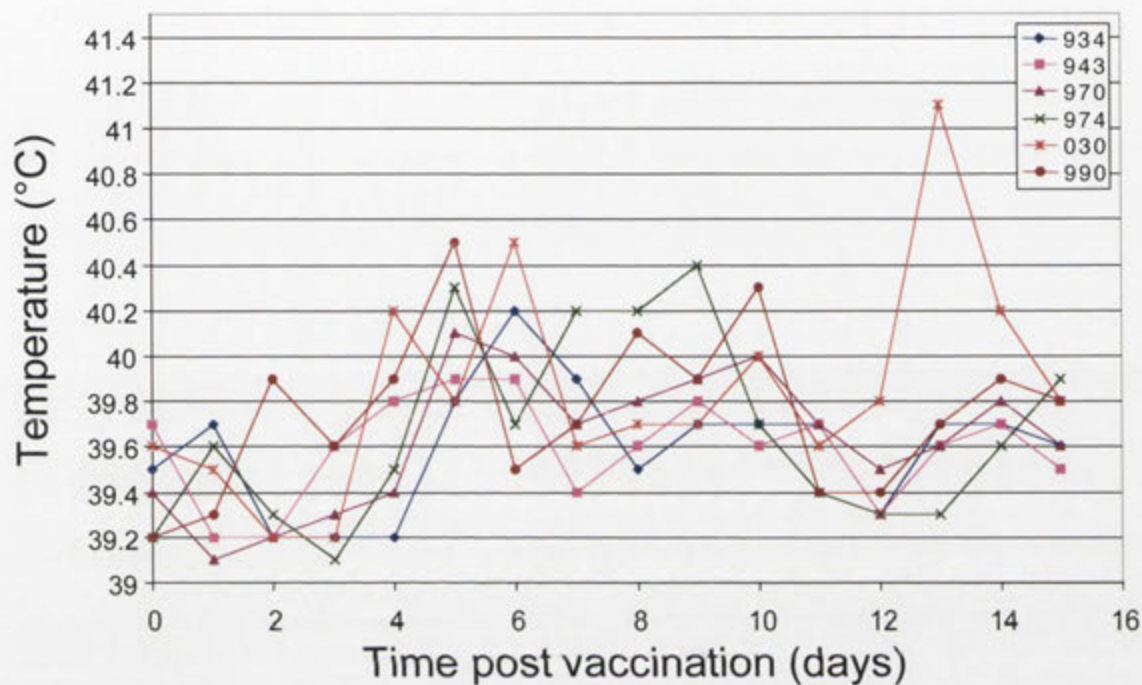
**Figure 4.19** - Rabbits 15 days after inoculation with 5000 pfu of Uriarra  $\Delta M-T7gpt$ . Primary lesions (A and B, arrowed). Secondary lesions on the face, ears and eyes (C and D, arrowed). One rabbit (030) had persistent symptoms and more secondary lesions than other rabbits (E and F, arrowed). Bars on images are 10 mm in size.

Temperatures for each Uriarra  $\Delta M$ -T7gpt-vaccinated rabbit were recorded daily. All rabbits except rabbit 943 had temperatures above 40°C for 1-4 days following inoculation, primarily between 4 and 10 dpi (Figure 4.20), and rabbit 030 had a temperature over 40°C at 13 and 14 dpi.

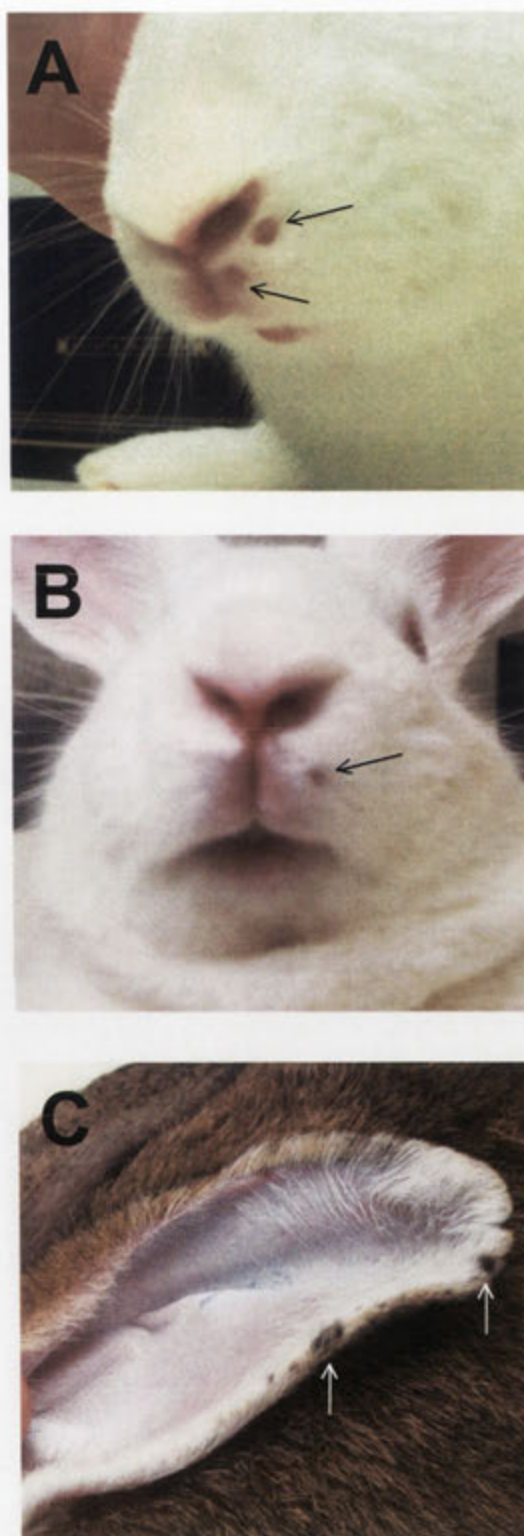
Thirty weeks following vaccination, scarring was seen at the sites of secondary lesions on most animals vaccinated with Uriarra  $\Delta M$ -T7gpt. Similar to Uriarra  $\Delta M10L/M11L$ gpt-vaccinated rabbits, this scarring was evident as patches where fur had not regrown over secondary lesions, as the ‘moth-eaten’ appearance of the fur on the edges of the ears, or fur regrowth of a different colour (Figure 4.21).

#### 4.2.3.4 Analysis of virus isolated from secondary lesions following vaccination with Uriarra $\Delta M$ -T7gpt

Biopsies of primary lesion and secondary lesion material were collected from rabbits 10 days following vaccination with Uriarra  $\Delta M$ -T7gpt, and used to infect RK13 cells *in vitro*. As described in Section 4.2.3.2, virus was grown without mycophenolic acid selection to permit the replication of both recombinant and wild type viruses. Viral DNA was isolated and examined by PCR to identify wild type and recombinant virus. Uriarra  $\Delta M$ -T7gpt-specific PCR product was isolated from most sites including secondary lesions on the eyelids, ears and anus, as indicated by the 603 bp M007-F1 x gpt-R1 PCR product amplified from viral DNA (Figure 4.22A). A Uriarra-specific, 542 bp M008-F1 x M007-RI PCR product was not amplified from any viral DNA sample, except the Uriarra DNA control (Figure 4.22B). These results indicate that secondary lesion formation was the result of dissemination of Uriarra  $\Delta M$ -T7gpt, and not due to a low-level contamination with wild-type Uriarra.

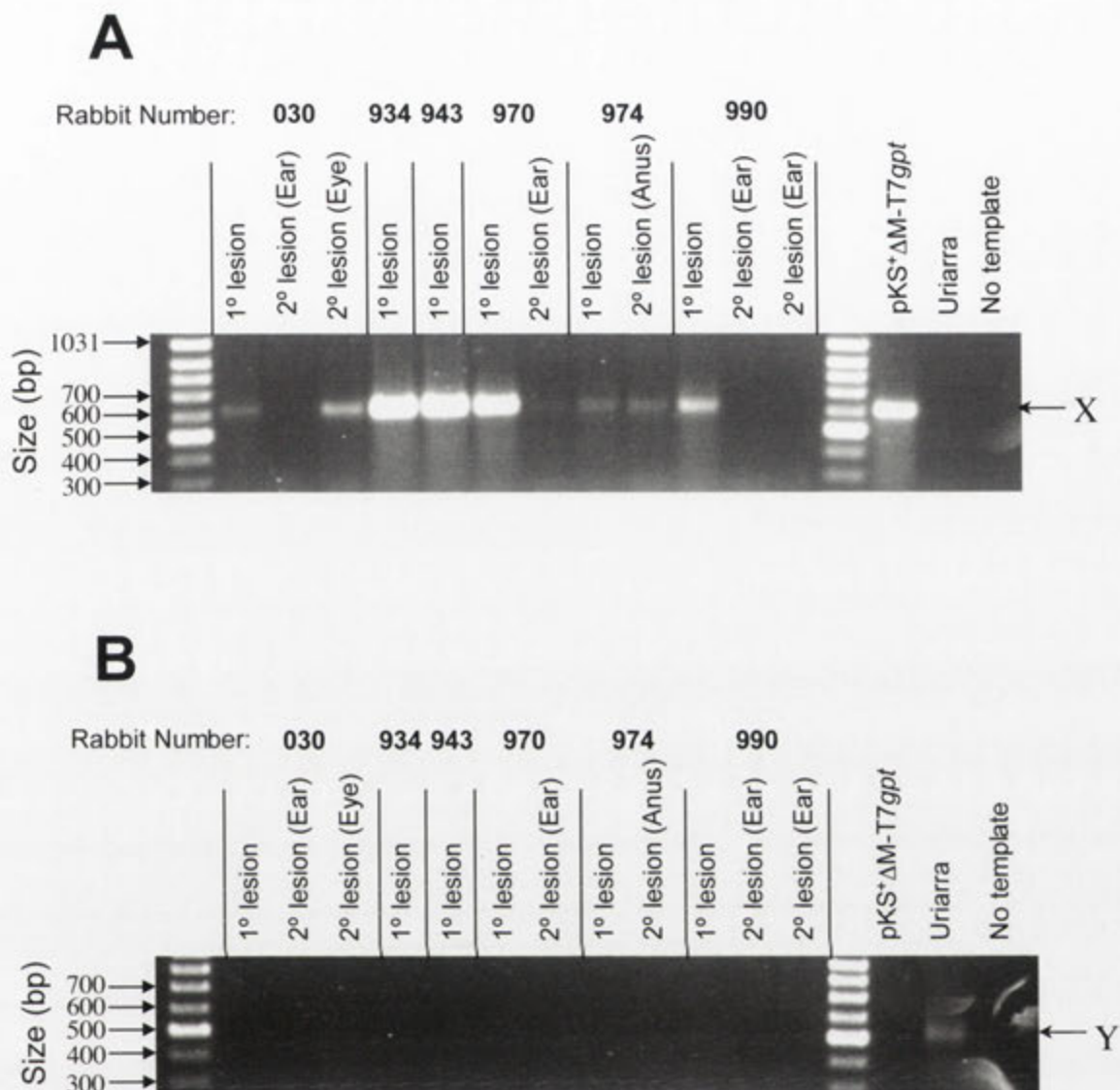


**Figure 4.20** - Rectal temperatures of rabbits following inoculation with 5000 pfu of Uriarra  $\Delta$ M-T7gpt.



**Figure 4.21** - Scarring on Uriarra  $\Delta$ M-T7gpt-vaccinated rabbits following vaccination. Scarring was evident on rabbits 30 weeks after vaccination at the sites of secondary lesions on the face and ears (A-C, arrowed).





**Figure 4.22 - PCR analysis of virus isolated from primary and secondary lesions of Uriarra ΔM-T7gpt-vaccinated rabbits.** DNA isolated from virus-infected cells seeded with tissue from a vaccinated rabbits (rabbits 030, 934, 943, 970, 974 and 990) taken 10 days after vaccination and analysed by PCR to identify recombinant virus (A) or wild type virus (B). The plasmid pKS<sup>+</sup>ΔM-T7gpt was used as a positive control for recombinant virus, and Uriarra genomic DNA was used as a positive control for wild type virus. A recombinant virus PCR product was amplified from most samples, including tissue collected from secondary lesions (panel A, 603 bp, arrow X). No wild type PCR product was amplified from any site (panel B, 542 bp, arrow Y).

#### 4.2.3.5 Vaccination of rabbits with TKO

Eighteen domestic male rabbits of between six and twelve months of age were inoculated with 5000 pfu of TKO and examined daily for 14 days. Vaccination experiments were performed in two batches. The first group of six rabbits responded very well to vaccination, so another twelve animals were inoculated with TKO in a second experiment. The clinical signs of all 18 rabbits are summarised in Table 4.3.

By 5 dpi, a lesion had developed at the inoculation site on 17 of the 18 rabbits, ranging in size from 8-15 mm in diameter (Figure 4.23). By 7 dpi, primary lesions were circumscribed and had increased in size on some rabbits with a range of 10-25 mm and were dark pink in colour (Figure 4.24). The lesions of some rabbits had darker centres or had scabbed over (Figure 4.24C and D), and by 9 dpi were covered with a scab. The lesions then regressed, fading to pale pink on most animals by 12 dpi (Figure 4.25). None of the 18 rabbits developed secondary lesions following vaccination.

The temperatures of TKO-vaccinated rabbits were recorded daily. None of the 18 animals had temperatures above 40°C at any point following inoculation with TKO (Figure 4.26).

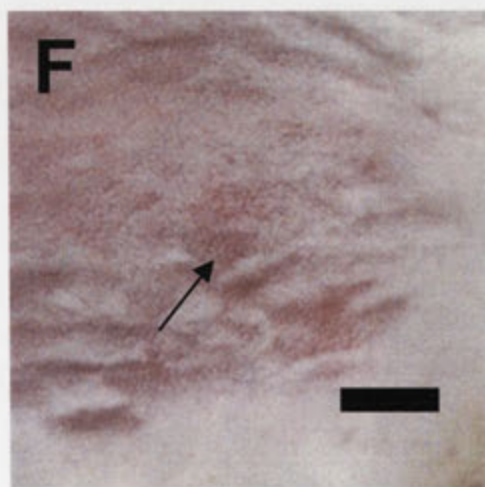
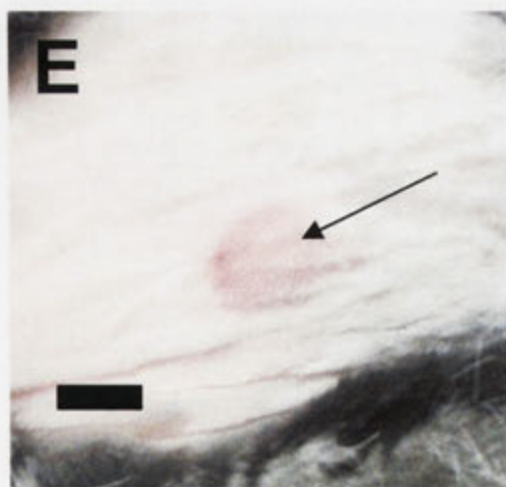
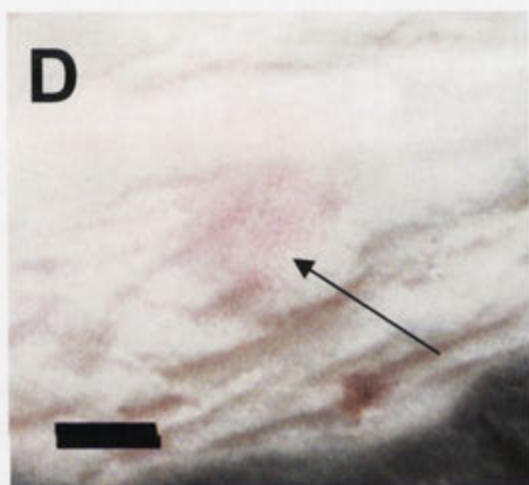
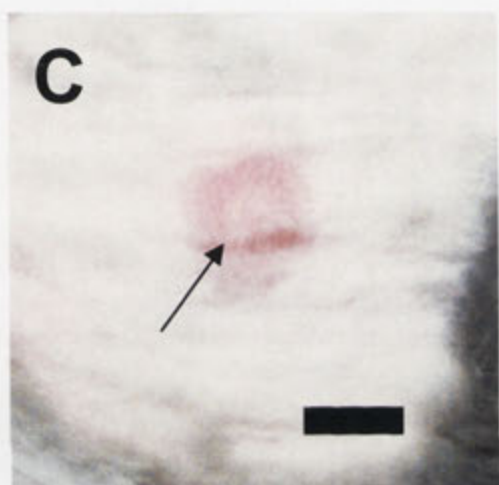
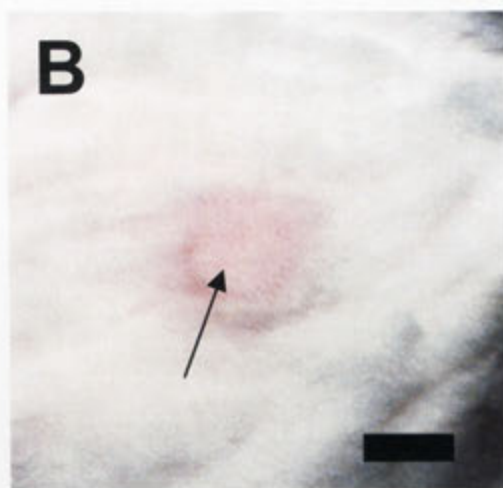
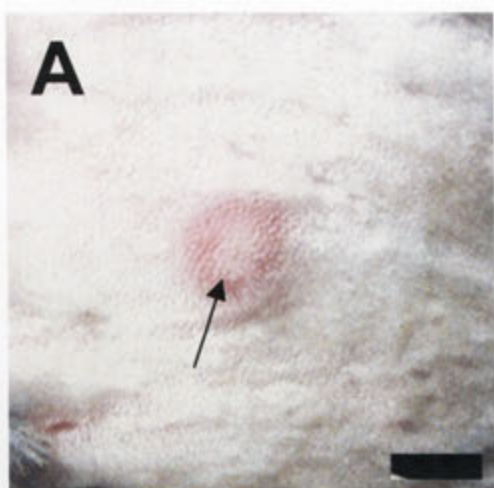
#### 4.2.4 Challenge of vaccinated rabbits with SLS

##### 4.2.4.1 Challenge of Uriarra $\Delta M10L/M11L$ gpt-vaccinated rabbits six weeks after vaccination

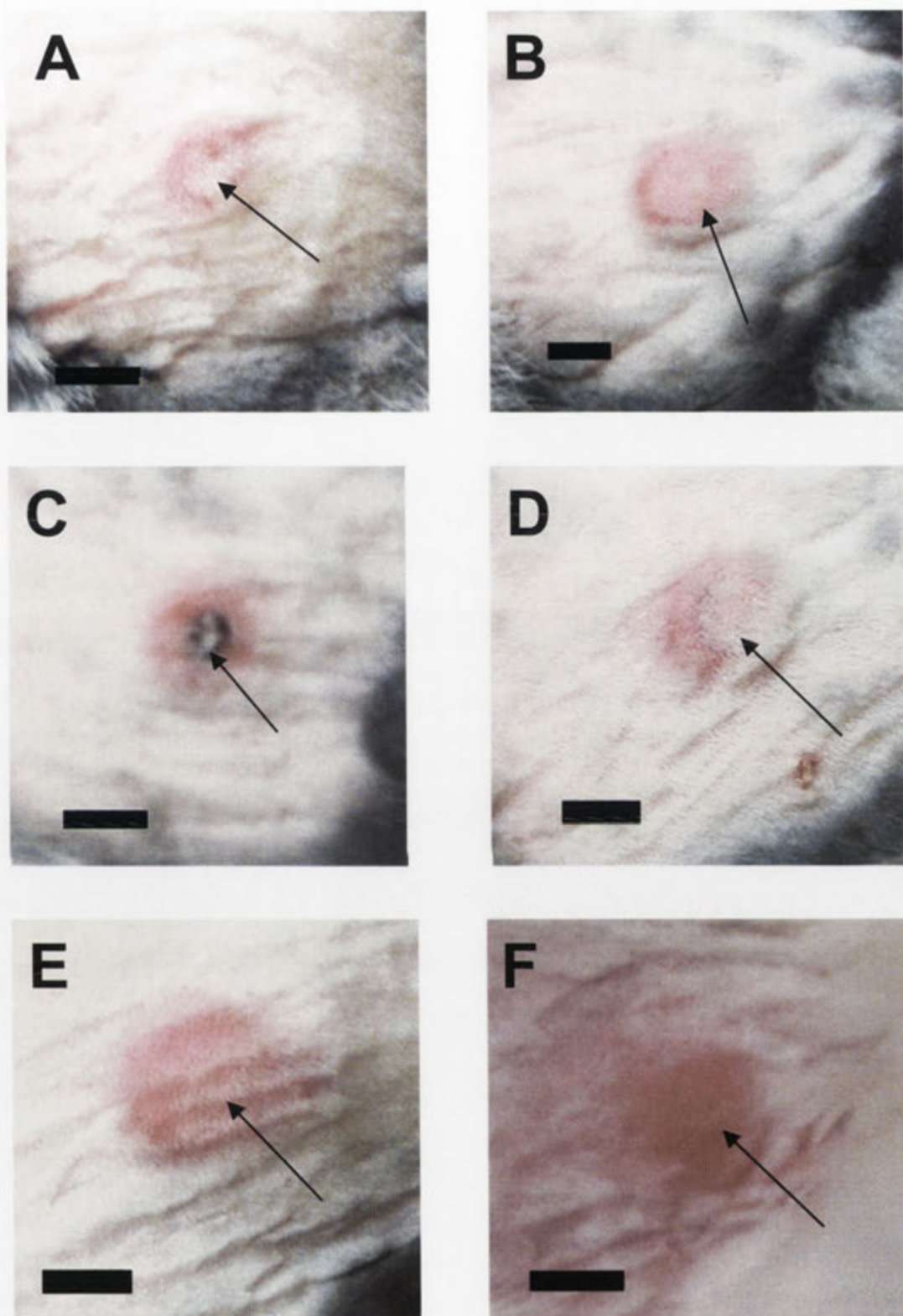
Six weeks after vaccination, rabbits were challenged with 1000 pfu of SLS subcutaneously on the right thigh. All animals developed a reddening of the skin at the challenge site by 1-2 days post challenge (dpc). This grew to up to 10 mm in diameter by 3-4 dpc (Figure 4.27). By 5 dpc, skin at the inoculation site had returned to normal.

**Table 4.3** – Clinical signs of mature domestic rabbits following vaccination with 5000 pfu of TKO.

Day post inoculation	Clinical signs
2	Slight reddening of skin at the inoculation site
4	Primary lesion is 8-15 mm in diameter, slightly raised and pink in colour.
6	Primary lesions circumscribed, 10-25 mm diameter
10	Primary lesion scabbed over on most animals, red or pink in colour.
14	Animals completely recovered. No secondary lesions were observed on any animal during infection

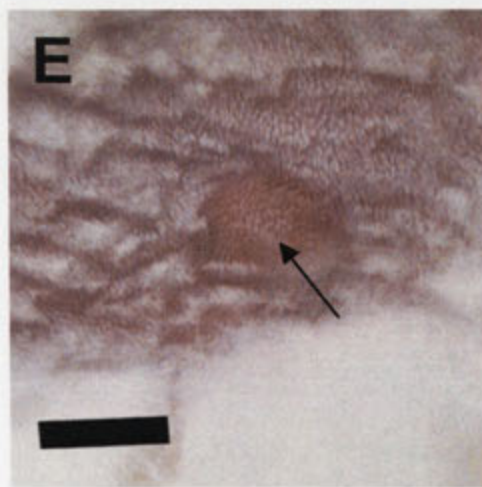
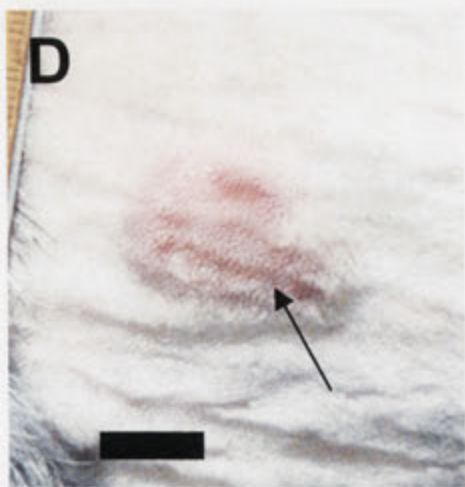
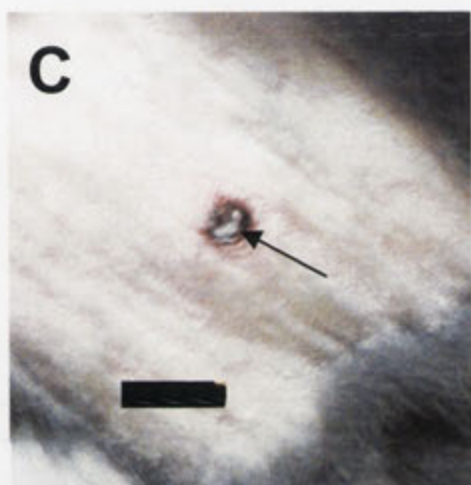
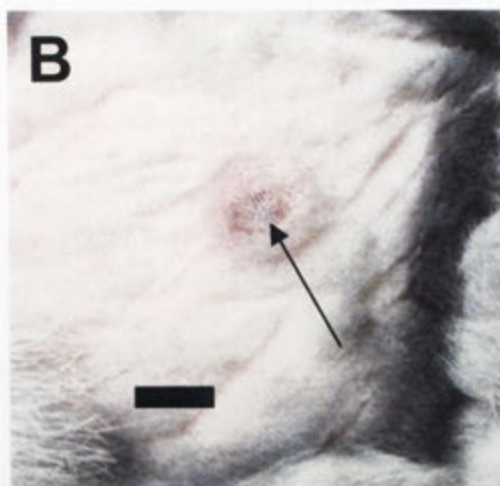
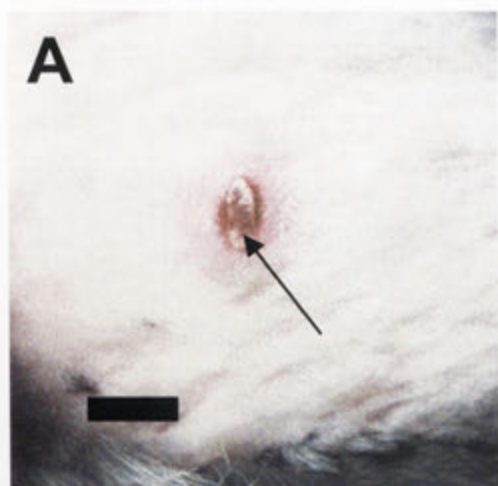


**Figure 4.23** - Inoculation site of rabbits 5 days after vaccination with TKO.  
Primary lesions are indicated with arrows. Bars on images are 10 mm in size.

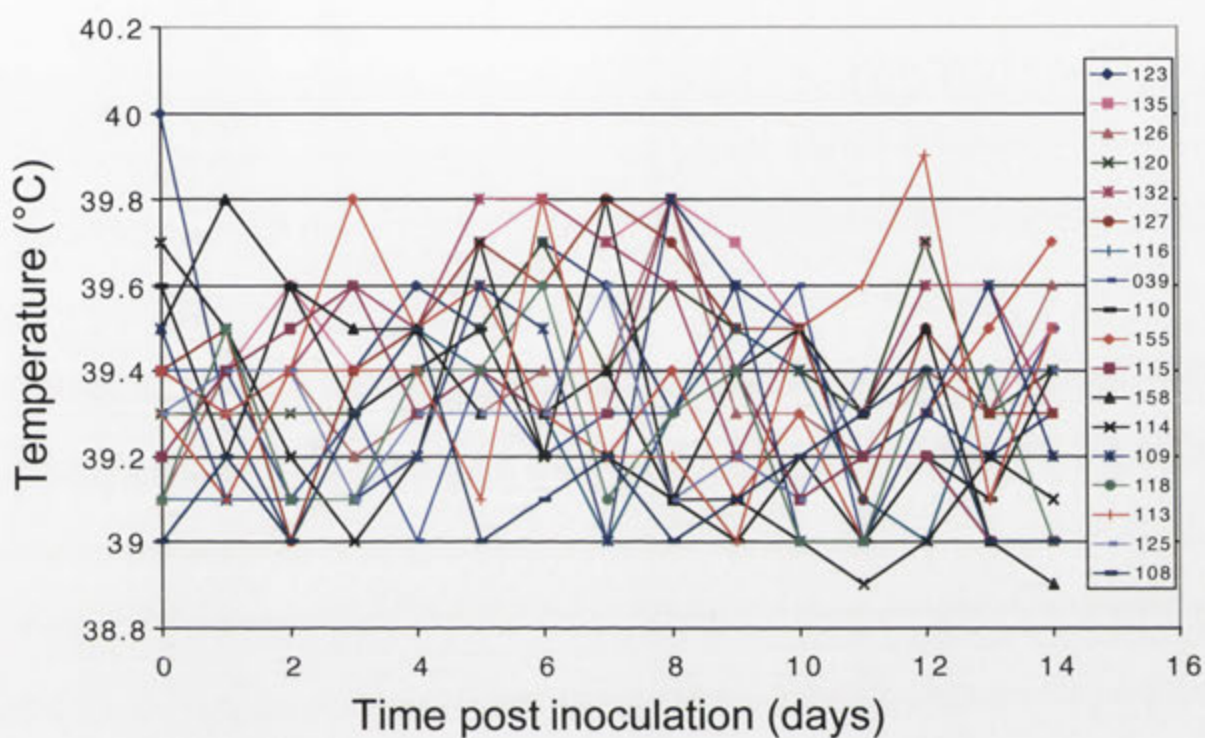


**Figure 4.24** - Inoculation site of rabbits 7 days after vaccination with TKO. Primary lesions were pink in colour and slightly raised (A-F, arrowed). The lesion of one animal had scabbed over (C). Bars on images are 10 mm in size.

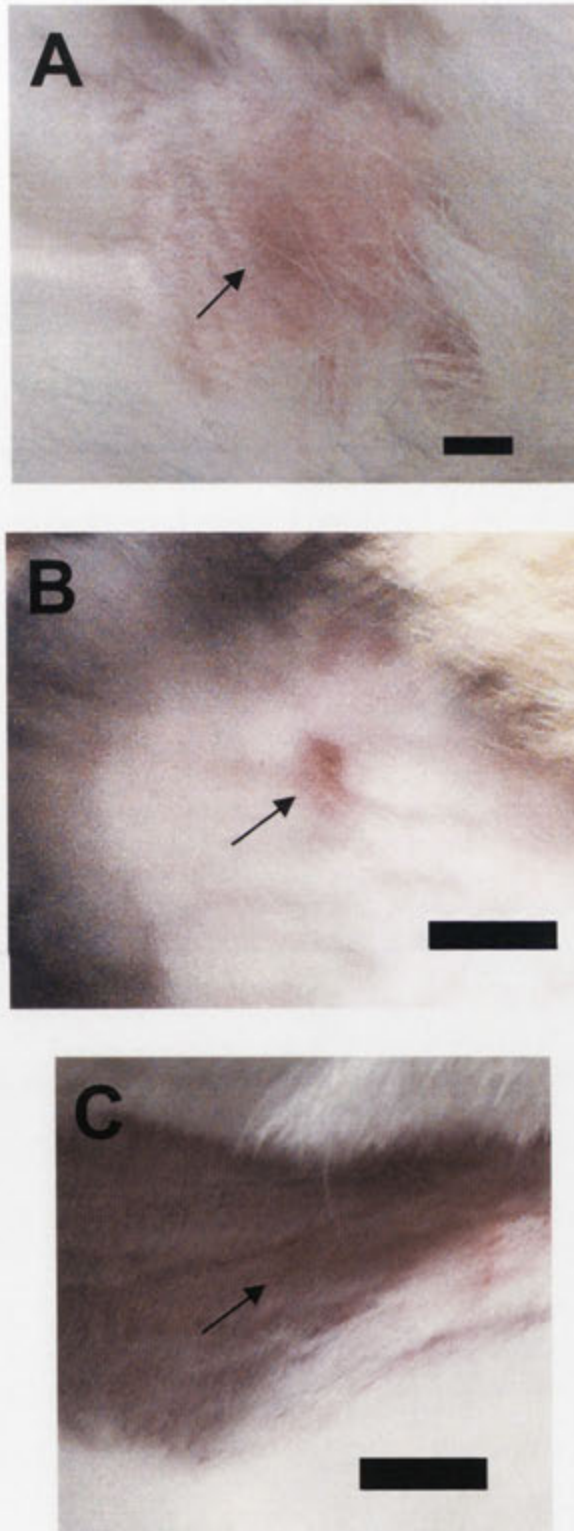




**Figure 4.25** - Inoculation site of rabbits 12 days after vaccination with TKO. Primary lesions are indicated with arrows. The primary lesions had scabbed over on several animals (A-C). Bars on images are 10 mm in size.



**Figure 4.26 - Rectal temperatures of rabbits following inoculation with 5000 pfu of TKO.**



**Figure 4.27** - Uriarra  $\Delta$ M10L/M11Lgpt-vaccinated rabbits 4 days following challenge with 1000 pfu of SLS. Rabbits had a reddening of the skin at the inoculation site (arrowed). Bars on images are 10 mm in size.



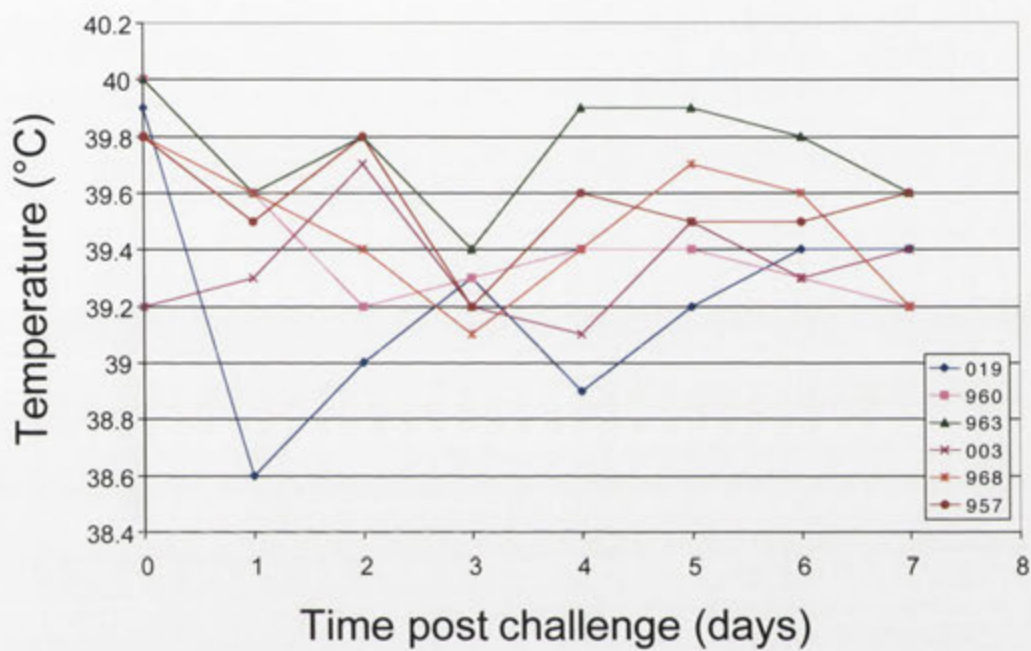
There was no large increase in temperature of the challenged animals, with the temperature of all animals staying below 40°C (Figure 4.28).

#### *4.2.4.2 Challenge of Uriarra $\Delta M10L/M11Lgpt$ -vaccinated rabbits 30 weeks after vaccination*

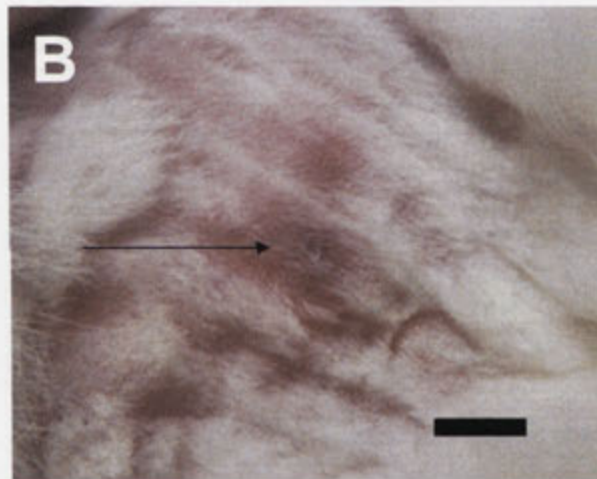
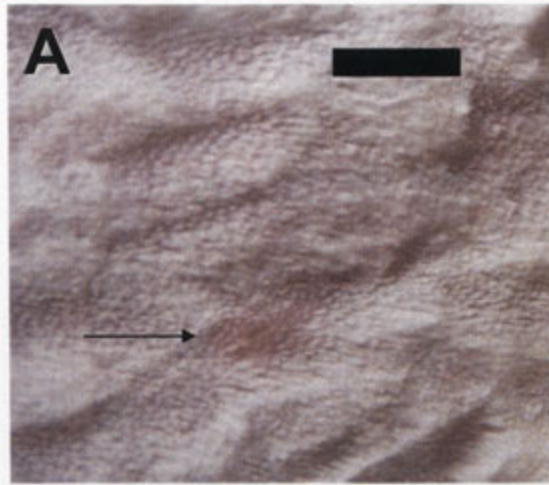
The same six rabbits previously challenged at 6 weeks were again challenged 30 weeks after vaccination with 1000 pfu of SLS delivered subcutaneously on the left thigh, and monitored for 1 week post challenge. Within 1-2 days, all animals had a reddening at the inoculation site ranging in size from 5-10 mm (data not shown). Some animals showed a slight thickening of the skin at the inoculation site, which regressed quickly. By 5 dpc, the inoculation site on all animals had returned to normal.

#### *4.2.4.3 Challenge of Uriarra $\Delta M-T7gpt$ -vaccinated rabbits six weeks after vaccination*

Rabbits that had been inoculated with Uriarra  $\Delta M-T7gpt$  were challenged with 1000 pfu of SLS subcutaneously on the right thigh. All animals developed a reddening of the skin at the inoculation site by 1-2 dpc. This was approximately 8 mm in diameter by 5 dpc (Figure 4.29A). By 6 dpc, skin at the inoculation site had returned to normal. Only one of the six rabbits developed a distinct lesion at the inoculation site (rabbit 943; Figure 4.29B). By 3 dpc, a hard red lump had developed at the inoculation site. This lump grew to 15 mm in diameter. The lesion was circumscribed began to darken and by 6 dpc was covered in a black scab. This rabbit had not developed any secondary signs of myxomatosis upon vaccination with Uriarra  $\Delta M-T7gpt$  (Section 4.2.3.3).



**Figure 4.28** - Rectal temperatures of Uriarra  $\Delta$ M10L/M11Lgpt-vaccinated rabbits following challenge with 1000 pfu of SLS.



**Figure 4.29** - Uriarra  $\Delta$ M-T7gpt-vaccinated rabbits 5 days following challenge with SLS. Transient reddening of the skin at the challenge site (A, arrowed). Rabbit 943 developed a distinct lesion at the inoculation site (B, arrowed). Bars on images are 10 mm in size.

There was no large increase in the temperature of challenged animals, with the temperature of all animals staying below 40°C, except for an early increase in temperature to 40°C at 1 dpc for two rabbits (970 and 974; Figure 4.30).

#### *4.2.4.4 Challenge of Uriarra $\Delta$ M-T7gpt-vaccinated rabbits 30 weeks after vaccination*

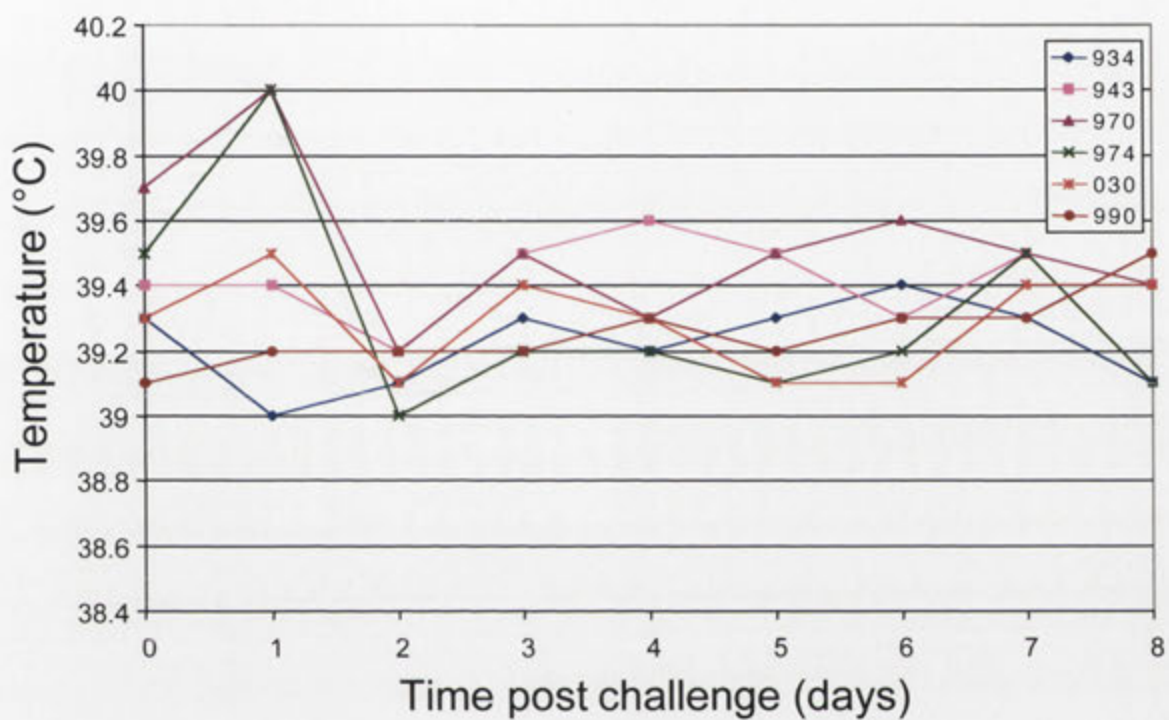
The Uriarra  $\Delta$ M-T7gpt-vaccinated rabbits were challenged again 30 weeks after vaccination with 1000 pfu of SLS delivered subcutaneously on the left thigh, and monitored for 1 week post challenge. Within 1-2 days, all animals exhibited a reddening at the inoculation site which faded quickly (data not shown). By 7 dpc, the inoculation site on all animals had returned to normal.

#### *4.2.4.5 Challenge of TKO-vaccinated rabbits six weeks after vaccination*

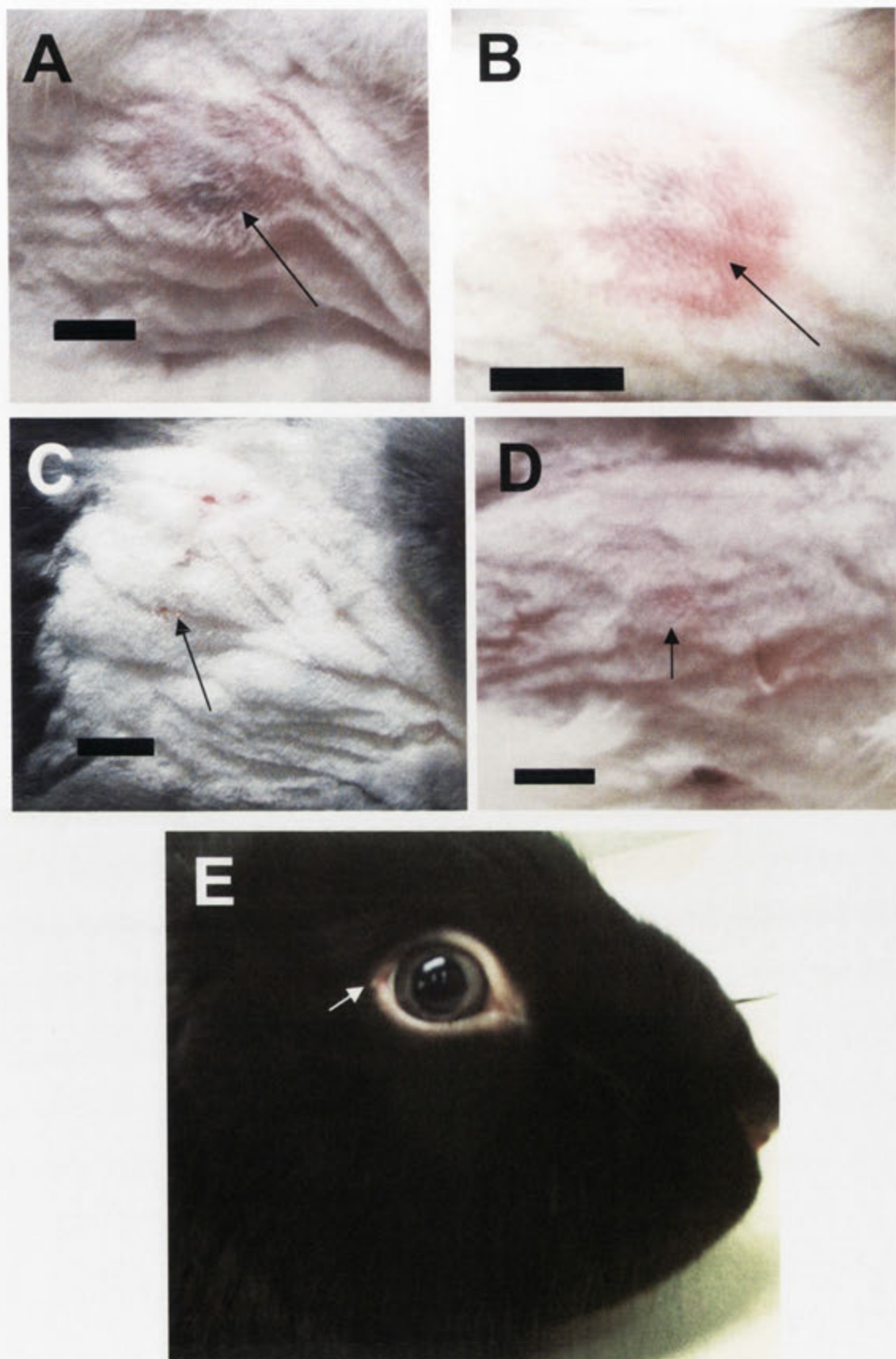
Following challenge with SLS, the 18 TKO-vaccinated rabbits could be divided into three groups based on the clinical signs displayed.

Six rabbits (110, 155, 114, 135, 132, 127) displayed a transient response at the inoculation site involving a reddening of the skin within 2 dpc, followed by fading and the skin returning to normal by 5 dpc (Figure 4.31C-D). This response was very similar to that observed when Uriarra  $\Delta$ M10L/M11Lgpt- or Uriarra  $\Delta$ M-T7gpt-vaccinated rabbits were challenged with SLS (Sections 4.2.4.1-4.2.4.4).

Eight rabbits (158, 116, 115, 109, 118, 125, 124, and 120) developed a distinct lesion at the inoculation site following challenge. These rabbits displayed a rapid reddening of the skin (within 1-2 dpc) at the inoculation site. However, instead of returning to normal, the skin thickened and developed into a red, protuberant lesion. The lesion at the inoculation site



**Figure 4.30** - Rectal temperatures of Uriarra  $\Delta M-T7gpt$ -vaccinated rabbits following challenge with 1000 pfu of SLS.



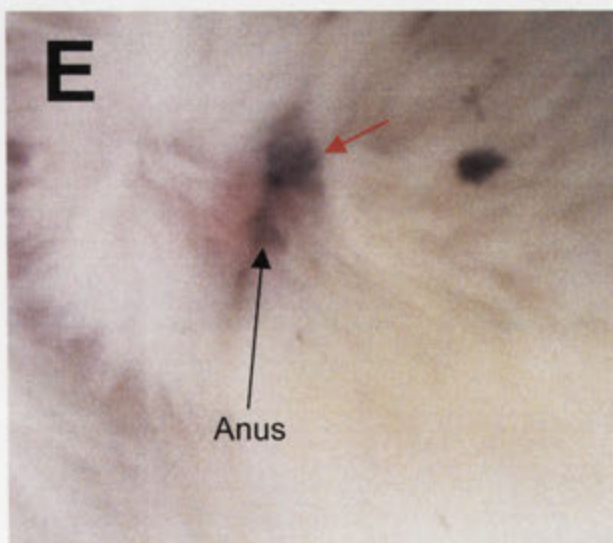
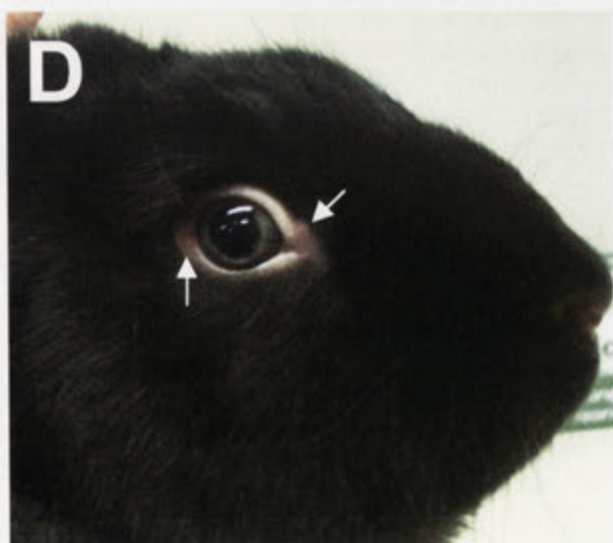
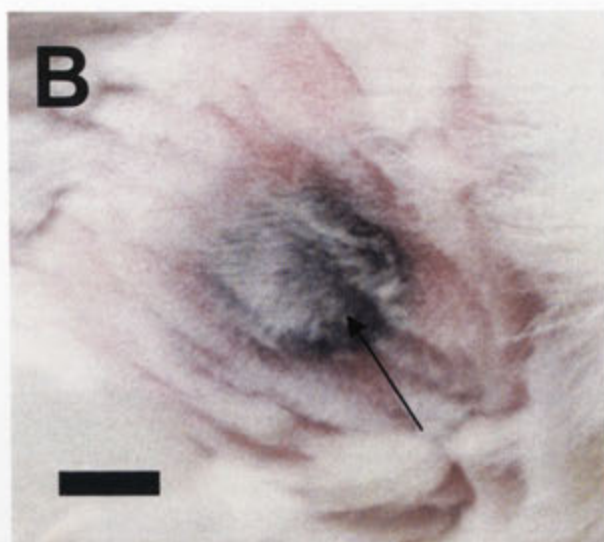
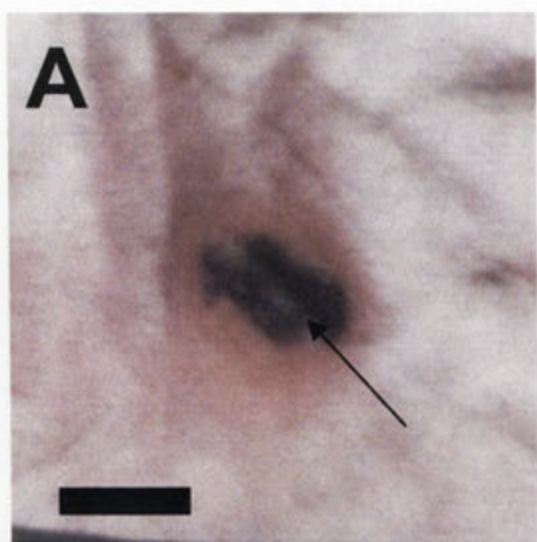
**Figure 4.31** - TKO-vaccinated rabbits 4 days following challenge with 1000 pfu of SLS. Primary lesions at the inoculation site (A and B, arrowed). Transitory reddening of the skin at the inoculation site (C and D, arrowed). A small secondary lesion on the eyelid (E, arrowed). Bars on images are 10 mm in size.



became circumscribed and scabbed over much more rapidly than during primary vaccination. By 4 dpc, haemorrhagic patches were visible on most lesions (Figure 4.31A), and all lesions were covered with a hard black scab by 6 dpc (Figure 4.32A and B).

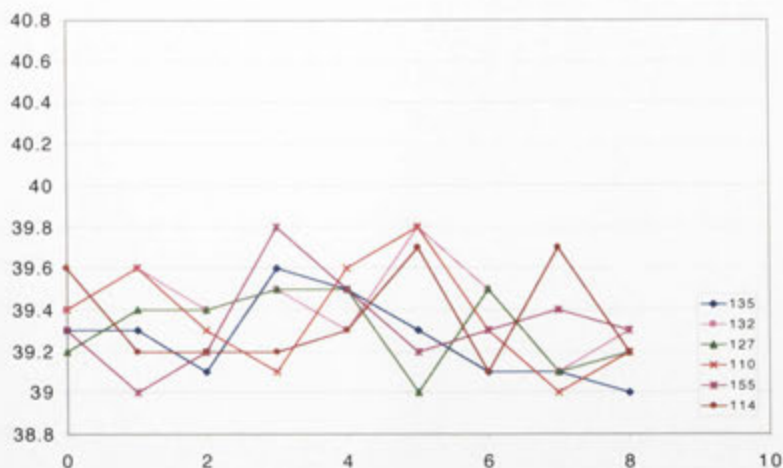
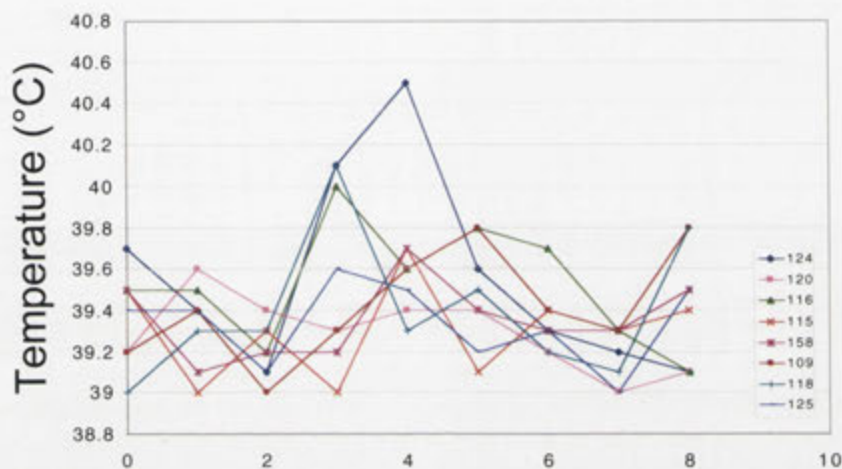
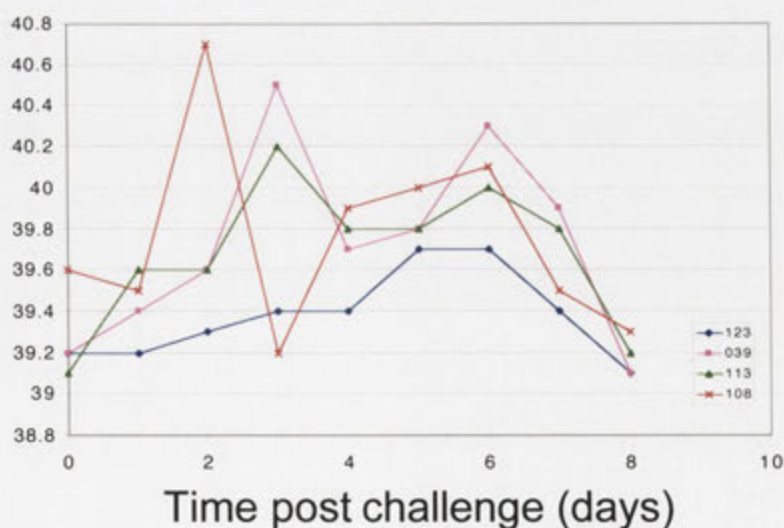
Four rabbits (113, 108, 039, and 123) developed a lesion at the inoculation site (Figure 4.31B), as observed in the previous group of rabbits, but also developed very small secondary lesions at sites including the eyelids, ears and anus (Figure 4.31E and Figure 4.32C-E). These secondary lesions were usually visible by 3-4 dpc - they appeared more rapidly than observed during infection with SLS in DNA-vaccinated rabbits (Chapter 3). However, the lesions also regressed or scabbed over very quickly, generally within 48 hours of appearance, and the rabbits had completely recovered from infection by 8-9 dpc. No more than 4 secondary lesions were observed on any one animal, and these were smaller than those seen on rabbits inoculated with Uriarra  $\Delta M10L/M11Lgpt$  or Uriarra  $\Delta M-T7gpt$ , being no larger than 5 mm in diameter.

Temperatures were recorded daily for all 18 rabbits and are shown in Figure 4.33. The temperatures have been divided into the three rabbit groups; those that did not develop a lesion (Figure 4.33A), those that developed a lesion at the inoculation site (Figure 4.33B) and those that developed secondary lesions (Figure 4.33C). Rabbits that did not develop a lesion had no increase in temperature above 40°C during the 8 days post challenge (Figure 4.33A). Three of the rabbits that developed a lesion at the inoculation site (rabbits 124, 116 and 118) had temperatures above 40°C for 1 or 2 days following challenge (Figure 4.33B). Three of the four rabbits that developed secondary lesions had temperatures above 40°C for 2 or 3 days following challenge (Figure 4.33C). Thus there appeared to be a correlation



**Figure 4.32** - TKO-vaccinated rabbits 7 days following challenge with 1000 pfu of SLS. Circumscribed and scabbed over primary lesions (A and B, arrowed). Small secondary lesions on the ear (C, arrowed), eyelid (D, arrowed) and anus (E, red arrow). Bars on images are 10 mm in size.



**A****B****C**

**Figure 4.33 - Rectal temperatures of TKO-vaccinated rabbits following challenge with 1000 pfu of SLS.** The temperatures of rabbits which upon challenge with SLS developed no lesion (A), a lesion at the inoculation site only (B) or secondary lesions (C) were monitored for 8 days post-challenge.

between the external clinical symptoms observed following challenge and the temperature of the animals.

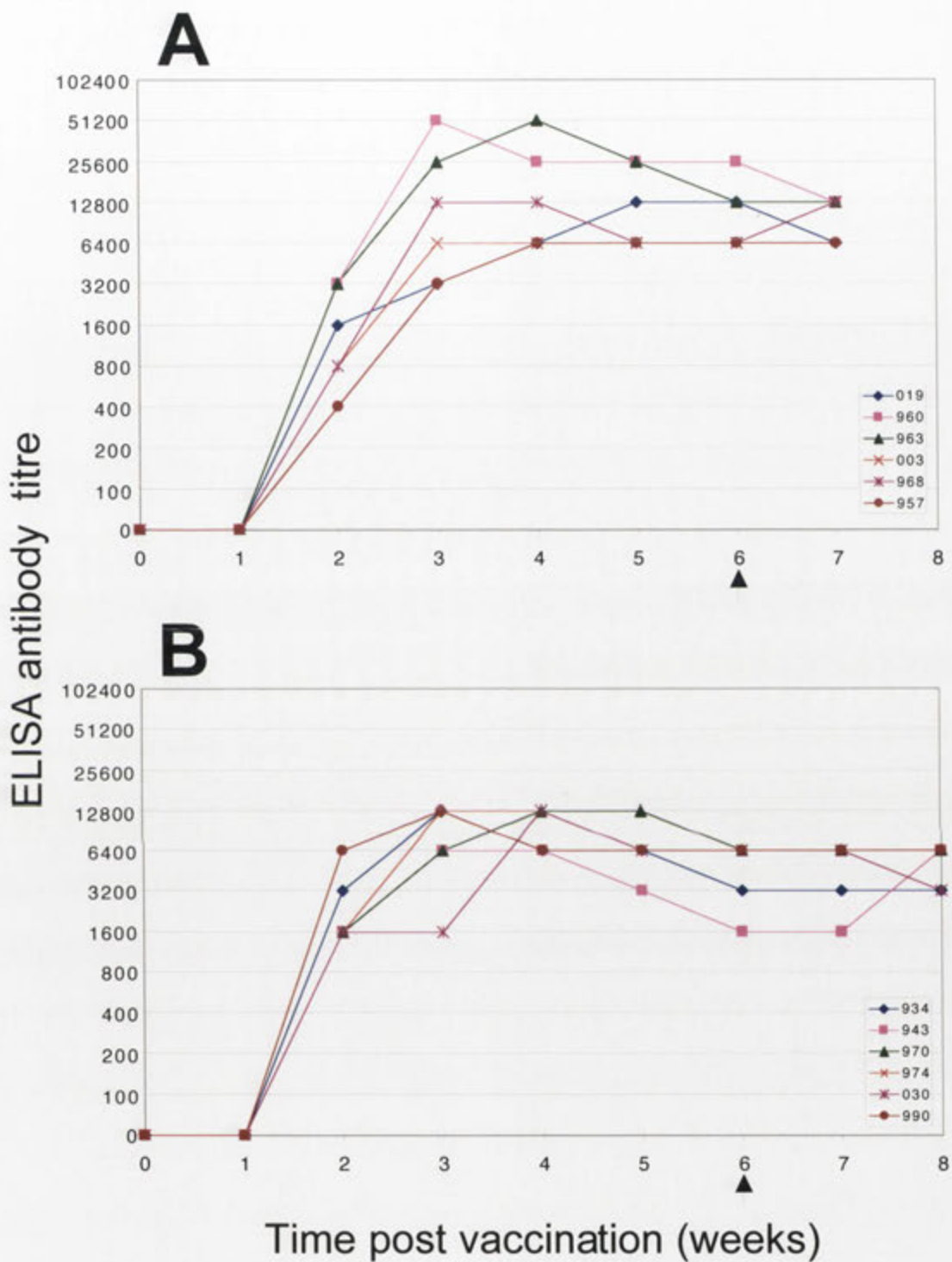
Due to space constraints in the PC2 animal facilities in which these experiments were conducted and the very high levels of protection seen amongst rabbits vaccinated with Uriarra  $\Delta$ M10L/M11Lgpt and Uriarra  $\Delta$ M-T7gpt at 30 weeks after vaccination, these 18 rabbits were not kept for re-challenge at 30 weeks.

#### **4.2.5 Analysis of anti-myxoma virus antibody development in vaccinated rabbits following vaccination and challenge**

##### *4.2.5.1 ELISAs*

The anti-myxoma virus ELISA antibody titre was determined for all serum samples after vaccination and after challenge with SLS using the method described in Kerr (1997). Anti-myxoma virus antibodies were first detected 14 days following inoculation with vaccine viruses. In rabbits vaccinated with Uriarra  $\Delta$ M10L/M11Lgpt, titres increased rapidly and ranged between 6400 and 51200 by 4 weeks post vaccination (wpv) and between 6400 and 25600 by 6 wpv (Figure 4.34A). Titres recorded in Uriarra  $\Delta$ M-T7gpt-vaccinated rabbits were not as high as those in Uriarra  $\Delta$ M10L/M11Lgpt-vaccinated rabbits. By 4 wpv, titres ranged between 6400 and 12800, falling slightly to between 1600 and 6400 by 6 wpv (Figure 4.34B).

Following challenge with SLS, there was no significant increase (4-fold or greater; Kerr, 1997) in antibody titre in any of the Uriarra  $\Delta$ M10L/M11Lgpt or Uriarra  $\Delta$ M-T7gpt vaccinated rabbits except for 943 (Figure 4.34). This rabbit showed a 4-fold increase in



**Figure 4.34** - ELISA anti-myxoma virus antibody titres in rabbits vaccinated with Uriarra  $\Delta$ M10L/M11Lgpt (A) or Uriarra  $\Delta$ M-T7gpt (B). The ELISA titre was determined by titration in duplicate of two-fold serial dilutions of rabbit sera from 1:100. The ELISA endpoint was defined as the reciprocal serum dilution 0.1 OD<sub>405</sub> units above the OD<sub>405</sub> of the 1:100 negative control. An OD<sub>405</sub> of less than 0.1 for a 1:100 dilution of serum was recorded as a titre of zero. Rabbits were challenged with SLS following sera collection at week 6 (arrows).

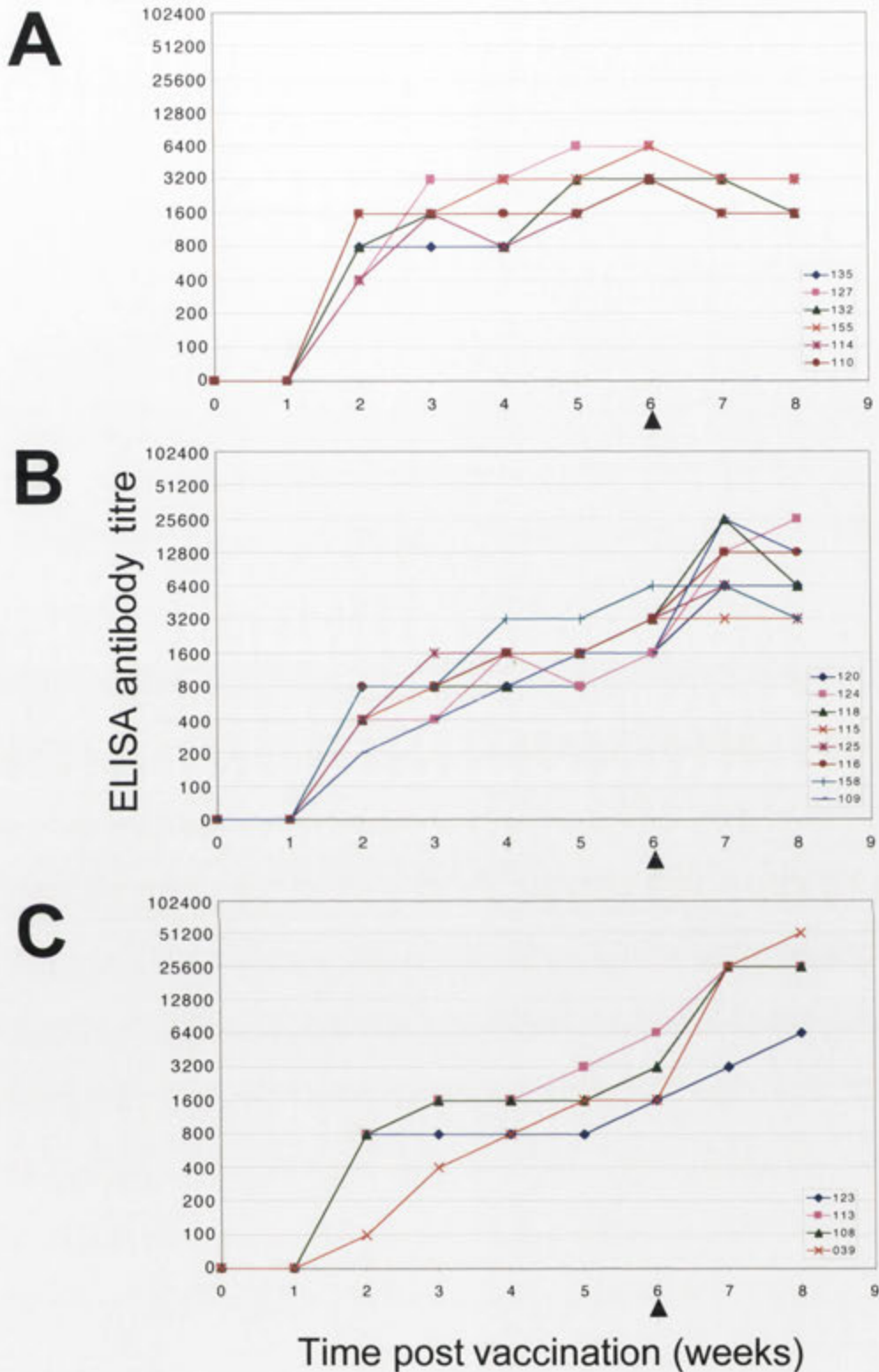
titre between 1 and 2 weeks following challenge. This rabbit was also the only rabbit in these groups that developed a lesion at the SLS inoculation site.

The ELISA titres for TKO-vaccinated rabbits have been divided into three graphs based on the symptoms exhibited by these rabbits following challenge with SLS (Figure 4.35). Following vaccination with TKO, anti-myxoma virus antibodies were first detected by ELISA 14 days post vaccination. Early titres (2-3 wpv) were generally lower than those measured in Uriarra  $\Delta$ M10L/M11Lgpt- and Uriarra  $\Delta$ M-T7gpt-vaccinated animals. However, by 6 wpv, titres were comparable with other vaccine groups with a range between 1600 and 6400 (Figure 4.35).

Following challenge, rabbits that did not develop a lesion showed no increase in antibody titre (Figure 4.35A). Of the eight rabbits which developed a lesion at the SLS inoculation site, four (124, 118, 116 and 109) showed a significant increase in titre following challenge (Figure 4.35B). All four rabbits which also developed secondary lesions after SLS infection had significant increases in anti-myxoma virus antibody titre following challenge, including rabbit 039 which showed a 32-fold increase in titre by 2 weeks after challenge (Figure 4.35C).

#### 4.2.5.2 Plaque reduction neutralisation assay (PRNA)

The ability of virus-specific antibody in the serum of TKO-vaccinated rabbits to neutralise myxoma virus *in vitro* was analysed using a plaque reduction neutralisation assay, as described in Kerr (1997). PRNA titres were not measured for Uriarra  $\Delta$ M10L/M11Lgpt- and Uriarra  $\Delta$ M-T7gpt-vaccinated rabbits because these vaccines were not considered suitable for further development. PRNA were conducted on serum from rabbits before



**Figure 4.35 - ELISA anti-myxoma virus antibody titres in rabbits vaccinated with TKO.** The ELISA titre was determined by titration in duplicate of two fold serial dilutions of rabbit sera from 1:100. The ELISA endpoint was defined as the reciprocal serum dilution 0.1 OD<sub>405</sub> units above the OD<sub>405</sub> of the 1:100 negative control. Rabbits were challenged with SLS following sera collection at week 6 (arrows). Rabbits which upon challenge with SLS developed no lesion (A), a lesion at the inoculation site only (B) or secondary lesions (C) have been graphed separately.

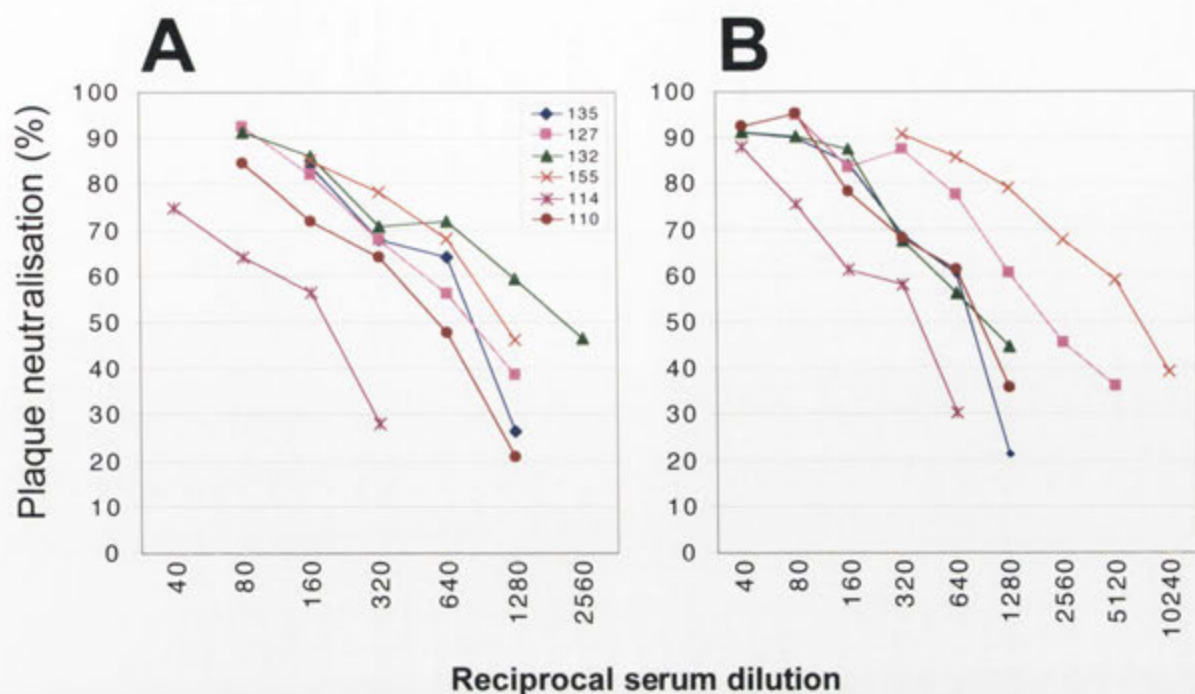
challenge (6 wpv) and two weeks after challenge (8 wpv). The percentage plaque neutralisation for each serum dilution tested and the corresponding 50% and 80% plaque neutralisation titres were recorded for rabbits that did not develop a lesion upon challenge (Figure 4.36), rabbits that developed a lesion following challenge (Figure 4.37) and rabbits that developed secondary lesions following challenge (Figure 4.38). At 6 wpv, 50% PRNA titres in TKO-vaccinated rabbits ranged between 80 and 1280 (Figures 4.36-4.38), except for rabbit 158 which had a titre of 10240 (Figure 4.37). There was no correlation between the pre-challenge PRNA titre and the severity of symptoms exhibited by TKO-vaccinated rabbits following challenge with SLS.

Following challenge, only one rabbit (155) amongst those that did not develop a lesion had a significant (4-fold or greater; Kerr, 1997) increase in PRNA titre (Figure 4.36), although this animal's ELISA titre fell slightly during this time (Figure 4.35A). A greater proportion of rabbits that developed a lesion at the inoculation site following challenge had increases in 50% PRNA titres, with five out of eight rabbits increasing titre by 8 to 64-fold, with final titres of between 320 and 10240 (Figure 4.37). Rabbit 158, which had the highest pre-challenge titre, showed a 16-fold decrease in 50% PRNA titre (Figure 4.37). All rabbits that developed secondary lesions upon challenge had significant increases in 50% PRNA titres, with 4- to 128-fold increases resulting in final titres of between 2560 and 10240 (Figure 4.38). There was no correlation between the magnitude of ELISA and PRNA titres increases following challenge with SLS.

#### **4.3 DISCUSSION**

This chapter has described the construction and testing of three candidate myxoma virus vaccines by the targeted inactivation of the viral immunomodulatory genes M010L, M011L

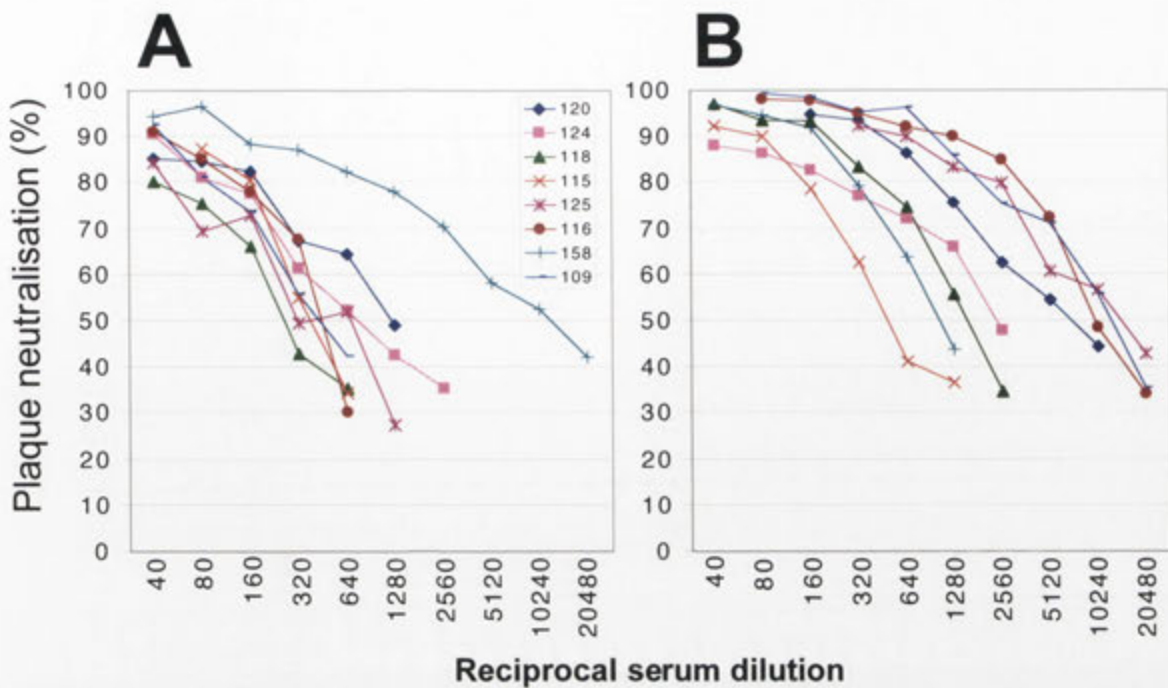




**C**

Rabbit	Neutralising antibody titre			
	Pre Challenge (Week 6)		Post Challenge (Week 8)	
	50%	80%	50%	80%
135	640	160	640	160
127	640	160	1280	320
132	1280	160	640	160
155	640	160	5120	640
114	160	< 40	320	40
110	320	80	640	80

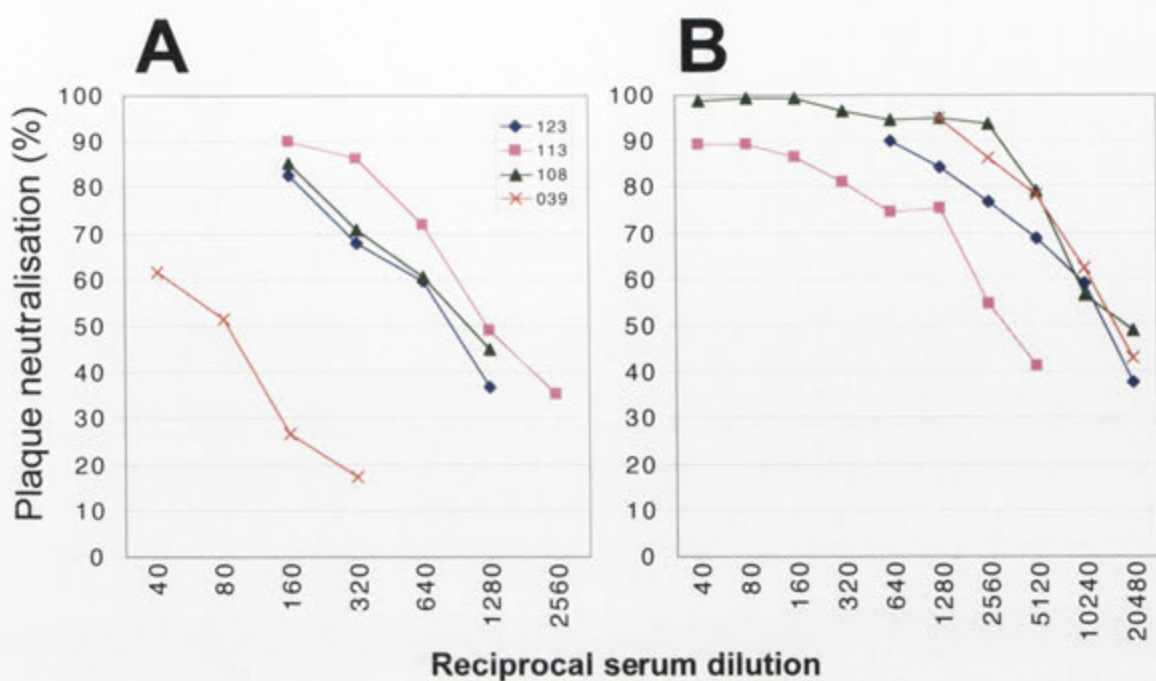
**Figure 4.36 - Analysis of neutralising anti-myxoma virus antibodies in the serum of rabbits vaccinated with TKO that did not develop a lesion following challenge with SLS.** Neutralising antibody was measured by plaque reduction neutralisation assay and the percentage plaque neutralisation recorded at week 6 (A) and week 8 (B) for each serum dilution (two-fold from 1:40 to 1:20480). The highest reciprocal serum dilutions at which 50% or greater and 80% or greater of myxoma virus plaque formation was neutralised is recorded (C).



C	Neutralising antibody titre			
	Pre Challenge (Week 6)		Post Challenge (Week 8)	
	50%	80%	50%	80%
Rabbit	50%	80%	50%	80%
120	640	160	5120	640
124	640	80	1280	160
118	160	< 40	1280	3200
115	320	80	320	80
125	160	40	10240	1280
116	320	80	5120	2560
158	10240	640	640	160
109	320	80	10240	1280

**Figure 4.37 - Analysis of neutralising anti-myxoma virus antibodies in the serum of rabbits vaccinated with TKO that developed a primary lesion only following challenge with SLS.** Neutralising antibody was measured by plaque reduction neutralisation assay and the percentage plaque neutralisation recorded at week 6 (A) and week 8 (B) for each serum dilution (two-fold from 1:40 to 1:20480). The highest reciprocal serum dilutions at which 50% or greater and 80% or greater of myxoma virus plaque formation was neutralised is recorded (C).





**C**

Rabbit	Neutralising antibody titre			
	Pre Challenge (Week 6)		Post Challenge (Week 8)	
	50%	80%	50%	80%
123	640	160	10240	1280
113	640	320	2560	320
108	640	160	10240	2560
039	80	< 40	10240	2560

**Figure 4.38** - Analysis of neutralising anti-myxoma virus antibodies in the serum of rabbits vaccinated with TKO that developed secondary lesions following challenge with SLS. Neutralising antibody was measured by plaque reduction neutralisation assay and the percentage plaque neutralisation recorded at week 6 (A) and week 8 (B) for each serum dilution (two-fold from 1:40 to 1:20480). The highest reciprocal serum dilutions at which 50% or greater and 80% or greater of myxoma virus plaque formation was neutralised is recorded (C).

and M007. These vaccines were evaluated for their acceptability and efficacy. Uriarra  $\Delta$ M10L/M11Lgpt and Uriarra  $\Delta$ M-T7gpt induced extremely mild myxomatosis in all vaccinated rabbits, which was not considered acceptable for use in pet animals. TKO was considered acceptable, with infection limited to a small lesion at the inoculation site only, with no secondary lesion development in the 18 rabbits vaccinated. All three vaccines conferred complete protection from lethal challenge. A small proportion (22%) of TKO-vaccinated rabbits developed very small secondary lesions upon challenge, but there were no clinical signs of myxomatosis observed in any of these animals.

The acceptability and efficacy of the three candidate vaccines, the role of measured virus-specific antibody in protection from myxoma virus challenge, and the possible role of myxoma virus EEV in the pathogenesis of Uriarra  $\Delta$ M10L/M11Lgpt have been discussed below.

#### **4.3.1 Acceptability versus efficacy in myxoma virus vaccines**

The three candidate vaccines have been evaluated for acceptability and efficacy. Both Uriarra  $\Delta$ M10L/M11Lgpt and Uriarra  $\Delta$ M-T7gpt were highly protective vaccines, with absolutely minimal symptoms of myxomatosis upon challenge with SLS up to 30 weeks after vaccination. Replication of SLS was extremely limited and cleared very rapidly, as exhibited by the transient reddening of the skin at the inoculation site (Fenner *et al.*, 1953). However, these viruses cannot be considered acceptable vaccines as they induced secondary lesions on vaccinated animals, and one of the Uriarra  $\Delta$ M-T7gpt-vaccinated rabbits developed a more persistent disease and exhibited some weight loss during vaccination. The secondary lesions, as well as potentially distressing for rabbit owners, are also sites where the virus could potentially be transmitted by arthropod vectors, increasing

the chances of the vaccine virus being transmissible. Also, most of the rabbits exhibited scarring at sites where secondary lesions developed, visible over 30 weeks post vaccination and likely to be permanent.

In contrast, TKO is a more acceptable vaccine in rabbits of this age group. The virus induced minimal symptoms in domestic rabbits, limited to a small primary lesion. However, the level of protection conferred by TKO was less than that of the more virulent vaccine viruses. Twelve out of eighteen TKO-vaccinated rabbits exhibited a lesion at the challenge site, and four of these also developed a limited number of very small secondary lesions, indicating SLS replication and dissemination was occurring. However, these symptoms are extremely minor compared to SLS infection in non-immune rabbits, such as those vaccinated with pDual2+ (Chapter 3), where the virus rapidly disseminated to secondary sites and was lethal within 13 days. In TKO-vaccinated rabbits, the SLS infection was controlled and cleared extremely rapidly, as exhibited by rapid circumscription and scabbing of the lesion at the inoculation site and secondary lesions, indicative of potent cell-mediated clearance of the virus in the skin. These post-challenge symptoms have been observed in previously infected and immune wild rabbits (Fenner *et al.*, 1953; Kerr, 1997).

Although TKO does not meet the ‘ideal’ standard for a myxomatosis vaccine established in Section 1.6, due to the appearance of secondary lesions on some rabbits upon lethal challenge, it appears that a vaccine which *does* confer this complete protection from infection (such as Uriarra  $\Delta$ M10L/M11Lgpt) would be too virulent to be acceptable. There appears to be a small window of opportunity where the virulence of a live myxoma virus

vaccine allows it to be both acceptable and effective enough to protect from more serious symptoms of myxomatosis. The TKO vaccine satisfies both of these criteria.

### **4.3.2 Antibody titres in vaccinated rabbits**

The severity of clinical signs observed in vaccinated rabbits following SLS challenge could not be predicted based on pre-challenge ELISA or PRNA titres. For example, pre-challenge neutralising antibody titres were comparable between groups of TKO-vaccinated rabbits that did not develop a lesion upon challenge and those that developed a lesion at the inoculation site or secondary lesions. This indicates that the level of anti-myxoma virus antibody following vaccination is not the key immune mechanism that confers protection.

A large proportion (77%) of those rabbits that developed distinct lesions at the inoculation site or secondary lesions, showed a significant increase in antibody levels following challenge. The development of a lesion is indicative of substantial viral replication in the skin at the inoculation site, and the subsequent production of viral proteins. A more vigorous immune response must be mounted against this infection, leading to the proliferation of myxoma virus-specific memory B and T cell populations and subsequent increase in myxoma virus-specific antibody titres. It has previously been found that only 50% of immune wild rabbits showed a significant increase in ELISA titre following challenge with virulent myxoma virus, despite the development of a lesion at the inoculation site (Kerr, 1997). The transient reddening of the skin following challenge observed on rabbits vaccinated with Uriarra  $\Delta M10L/M11Lgpt$ , Uriarra  $\Delta M-T7gpt$  and some rabbits vaccinated with TKO, is indicative of very limited viral replication that is rapidly cleared from the skin, most likely by virus-specific cell-mediated immune

responses. The limited amount of viral antigens produced did not induce the amnesiastic antibody response observed in other animals.

### **4.3.3 Role of EEV particles in myxoma virus pathogenesis**

Although Uriarra  $\Delta M10L/M11Lgpt$  cannot productively infect lymphocytes as indicated by its *in vitro* characteristics in RL-5 cells (Section 4.2.2.2), it was able to disseminate and cause secondary lesions at distal sites. Therefore, it appears this virus has the ability to disseminate to secondary sites outside of infected lymphocytes. The pathology of Uriarra  $\Delta M10L/M11Lgpt$  in infected rabbits was more severe than that reported for Lausanne $\Delta M010L/M011L$ , which did not induce any secondary lesions (Opgenorth *et al.*, 1992).

The comet plaque morphology of Uriarra  $\Delta M10L/M11Lgpt$  plaques on Vero cells (Figure 4.13) suggests that this virus is producing more EEV particles than Uriarra. This plaque morphology is caused by the release of EEV particles from infected cells and their distribution to distant sites on the cell monolayer by convection currents in the media (Law *et al.*, 2002). The increased dissemination of Uriarra  $\Delta M10L/M11Lgpt$  observed *in vivo* compared to that reported for Lausanne $\Delta M010L/M011L$  may be due to enhanced production of EEV particles from Uriarra  $\Delta M10L/M11Lgpt$ -infected cells. There is no published information about the role of EEV particles in the pathogenesis of myxoma virus, although they are critical for the dissemination of VV to distal tissues in the VV/mouse model (Payne, 1980; Payne and Kristensson, 1985; Smith and Vanderplasschen, 1998; Smith *et al.*, 2002).

The cause of this phenotype is currently unknown. M010L and M011L and their homologues in other poxviruses have no reported effects on EEV production or plaque phenotype. The inactivation of M010L and M011L in Lausanne was mediated by the insertion of the *E. coli*  $\beta$ -galactosidase gene in the overlapping region of the two genes, with no myxoma virus sequence deleted (Opgenorth *et al.*, 1992). The deletion in Uriarra  $\Delta$ M10L/M11Lgpt may have affected promoter and regulatory elements controlling the neighbouring gene M009L. The function of this gene is unknown. M009 contains several kelch-ring motifs (Cameron *et al.*, 1999), and kelch-like proteins in VV have demonstrated effects on the formation of VV-induced cellular projections that are associated with EEV particles (Pires de Miranda *et al.*, 2003). It is interesting to note that highly virulent field strains of myxoma virus have been isolated in Australia with deletions in the M009L gene (Peter Kerr, personal communication), suggesting a possible role for this gene in viral pathogenesis. Further investigation of Uriarra  $\Delta$ M10L/M11Lgpt and the possible role of M009L and EEV in myxoma virus pathogenesis are certainly warranted.

#### 4.3.4 Conclusions

Of the three candidate vaccines tested, only TKO can be considered acceptable. The appearance of secondary lesions on almost all rabbits vaccinated with Uriarra  $\Delta$ M10L/M11Lgpt and Uriarra  $\Delta$ M-T7gpt would not lead to an acceptable vaccine. Although only six animals were vaccinated with each of the viruses in this trial, the potential complication rate of such vaccines in younger and immunocompromised animals would most likely be unacceptable. As TKO was the only one of the three candidate vaccines to pass the initial selection criteria of acceptability and efficacy, it was selected for further analysis and evaluation, as described in Chapters 5 and 6.

## **Chapter 5**

# **The pathogenesis of TKO in domestic and wild rabbits**

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## CHAPTER 5 – THE PATHOGENESIS OF TKO IN DOMESTIC AND WILD RABBITS

### 5.1 INTRODUCTION

The three deletion mutant vaccines evaluated in Chapter 4 all conferred excellent protection from challenge with myxoma virus. However, only TKO conferred this protection without causing any secondary lesions or visible scarring on vaccinated rabbits. As the candidate vaccine with the least vaccine-induced side effects combined with good protection from challenge, the pathogenesis of TKO in domestic rabbits was studied to further evaluate TKO's acceptability as a vaccine.

Histological analysis of skin lesion and draining lymph node sections allowed us to determine what level of tissue damage was occurring in TKO-infected rabbits. The measurement of titres of virus in the skin of domestic rabbits allowed us to infer the potential transmissibility of the TKO vaccine from a recently vaccinated domestic rabbit. This is because transmissibility of myxoma virus by arthropod vectors is directly related to the titre of virus in the skin (Fenner *et al.*, 1956). The titre of TKO in distal tissues was also measured to evaluate the *in vivo* dissemination of the vaccine virus.

Myxoma virus replicates to high titres in the testes of male rabbits and the resultant inflammatory reactions in the testes can make males infertile (Fountain *et al.*, 1997; Sobey and Turnbull, 1956). If TKO is to be utilised as a vaccine for farmed rabbits and commercial rabbit breeders, it must not adversely affect the fertility of male breeders. In the work described here, the amount of virus in the testes of infected rabbits was measured, and the testes examined histologically to determine if there were any changes that were indicative of reduced fertility.



The pathogenesis of TKO in wild rabbits was also studied. Measurement of TKO titres in wild rabbit skin allowed us to infer the potential transmissibility of the virus between wild rabbits, and its possible establishment in wild rabbit populations. The pathology of TKO infection in wild rabbit skin and draining lymph nodes was also examined.

## 5.2 RESULTS

### 5.2.1 Pathogenesis of TKO in mature domestic rabbits

TKO virus (5000 pfu) was inoculated subcutaneously into the dorsum of the right hind foot of mature domestic rabbits (6-15 months old). At each of 2, 4, 6, 10 and 20 days after infection, a group of three rabbits was killed. Samples of skin from the inoculation site, the right popliteal lymph node (draining lymph node), the left popliteal lymph node (contralateral lymph node) and right testis were removed. Half of the tissue was fixed in 10% formalin in PBS and prepared for histological analysis (haematoxylin and eosin staining), while half was frozen immediately on dry ice and used to determine the titre of virus in the tissue. This was the same methodology used in previous pathogenesis studies of Uriarra (Best *et al.*, 2000; Best and Kerr, 2000), allowing the use of data and histological sections from those experiments to be compared to those obtained here.

#### 5.2.1.1 Titre of TKO in domestic rabbit tissues

The skin from the inoculation site, the draining lymph node, the contralateral lymph node and testis were processed as described in Section 2.6.6, and the virus titres in these tissues determined by plaque assay, with a detection limit of  $10^2$  pfu per gram of tissue (pfu/g). Figure 5.1 shows the virus titre for each rabbit in the skin at the inoculation site, the draining lymph node and the contralateral lymph node. Samples where a trace of virus (a single plaque on one of duplicate samples) was found were defined as having a titre of 50

pfu/g ( $10^{1.69}$ ), and samples with no detectable virus were defined as having a titre of 0 pfu/g. The mean titres of Uriarra determined by Best and Kerr (2000) have been included in Figure 5.1 to allow comparison of TKO to a virus that is readily transmissible and disseminates to distal tissues, causing severe symptoms of myxomatosis in domestic rabbits.

TKO was detected at the inoculation site in all three rabbits at 2 days post inoculation (dpi), with titres ranging from a trace to  $9.4 \times 10^5$  pfu/g (Figure 5.1A). Maximum TKO titres were detected at 6 dpi, with a mean titre of  $1.4 \times 10^7$  pfu/g (range of  $3.5 \times 10^6$  to  $4.2 \times 10^7$  pfu/g). Two of the three rabbits had titres above  $10^7$  pfu/g, the titre of virus in the skin required for mosquito transmission of the virus (Fenner *et al.*, 1956). By 10 dpi, the mean virus titre had dropped to  $1 \times 10^3$  pfu/g (range of  $5.8 \times 10^2$  to  $3 \times 10^3$  pfu/g); virus was undetectable by plaque assay at 20 dpi (Figure 5.1A). In contrast, Uriarra had a peak mean titre of  $4 \times 10^8$  pfu/g at 6 and 10 dpi, and at 20 dpi, mean Uriarra titres in the skin were still  $10^6$  pfu/g (Figure 5.1A).

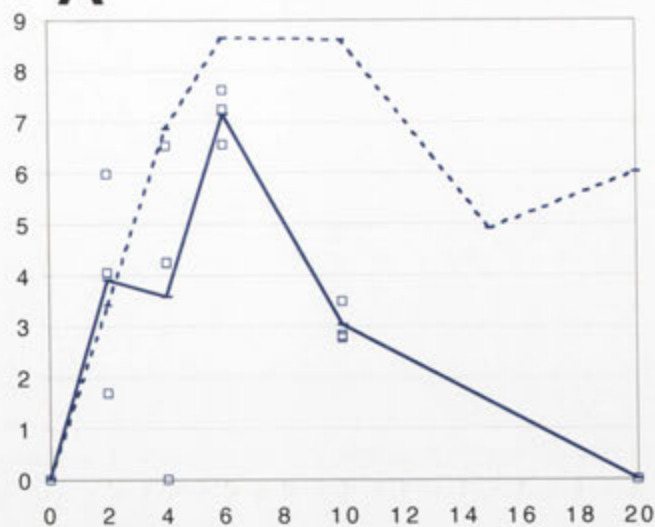
TKO was detected in the draining lymph node by 2 dpi with a mean titre of  $3.2 \times 10^2$  pfu/g (Figure 5.1B). Peak TKO titres were recorded at 4 dpi with a mean titre of  $1.1 \times 10^3$  pfu/g. TKO was still detectable in the draining lymph node at 10 dpi in two of three rabbits. TKO was not detected at 20 dpi. Previously determined Uriarra titres in the draining lymph node of domestic rabbits were 100-1000 times higher than TKO titres at 4, 6 and 10 dpi (Figure 5.1B).

Titres of TKO were lower in the contralateral lymph node than the draining lymph node, and generally at or just above the limit of detection ( $10^2$  pfu/g). TKO was first detected at 4

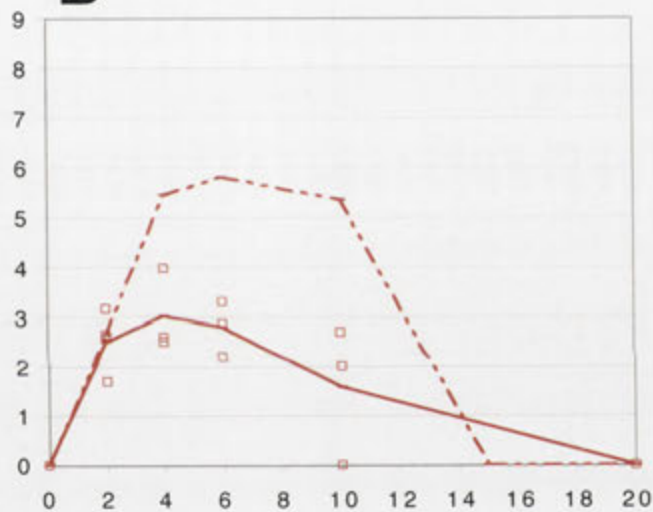
**Figure 5.1 - Myxoma virus titres in domestic rabbit tissues following inoculation with 5000 pfu of TKO.** Virus titres at the inoculation site (A), draining lymph node (B) and contralateral lymph node (C) were measured by plaque assay, with a detection limit of  $10^2$  pfu/g. Each open square represents an individual rabbit infected with TKO and killed 2, 4, 6, 10 or 20 dpi. A titre of zero indicates no virus was detected in the given tissue sample. A titre of  $10^{1.69}$  pfu/g indicates that a single plaque (a trace) was detected on duplicate sample wells. The solid line on each graph represents the geometric mean titre for the three rabbits sampled at each time point. The dotted line represents the geometric mean titre of Uriarra (of 2 or 3 rabbits) in the same tissues following inoculation with 100 pfu of virus, as determined by Best and Kerr (2000).

Virus titre ( $\log_{10}$ [pfu/g tissue])

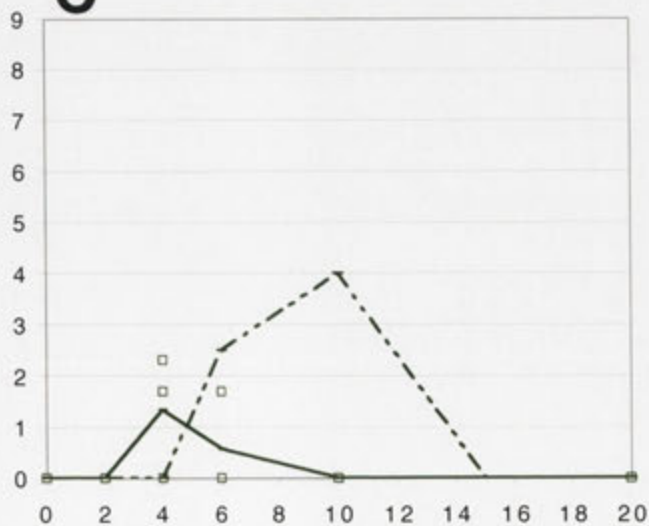
**A**



**B**



**C**



Time post infection (days)

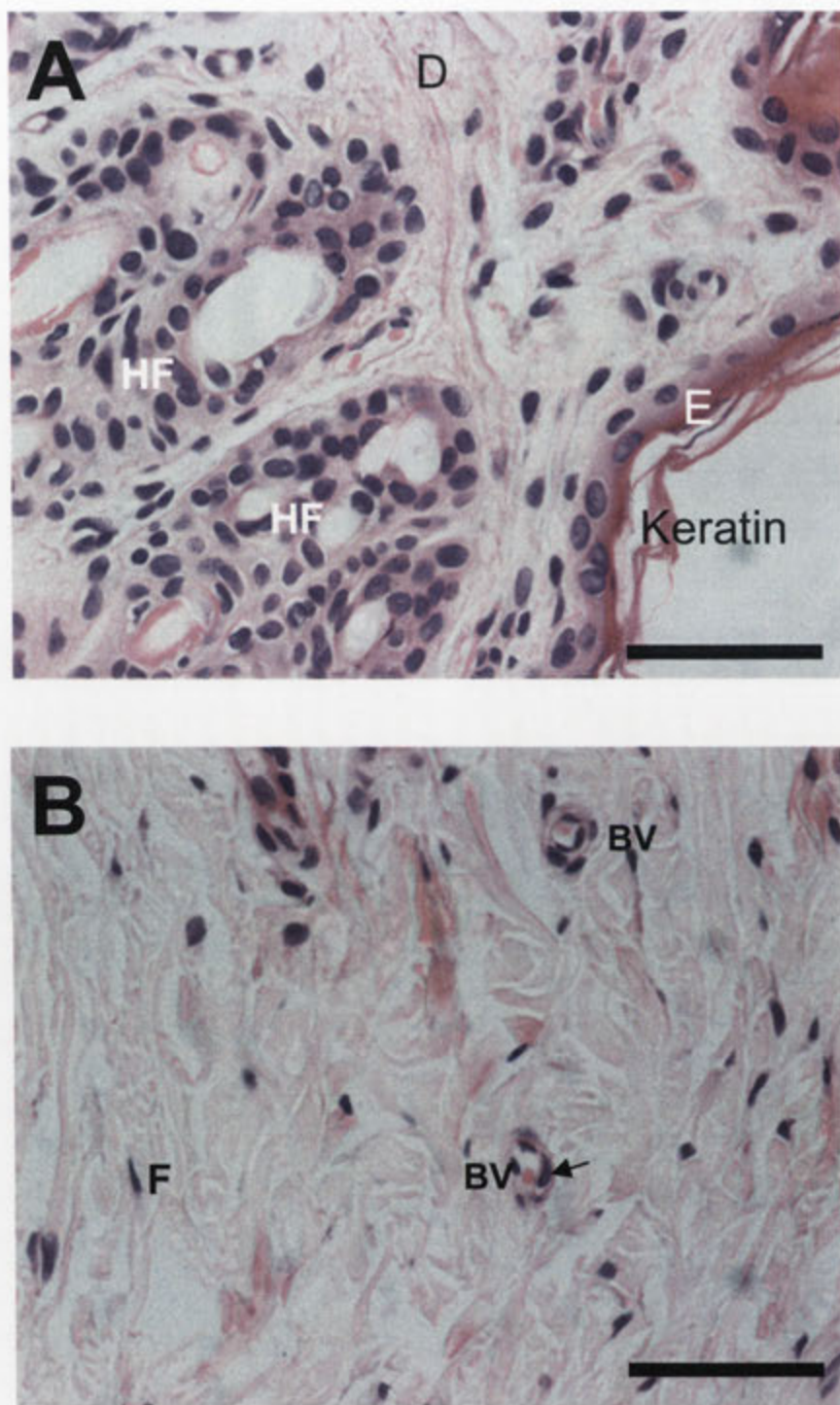
dpi in two of three rabbits, with a trace of virus in one animal and a titre of 200 pfu/g in the other (Figure 5.1C). By 6 dpi, a trace of virus was detected in only one of three rabbits, and virus was not detected in the contralateral lymph node at 10 or 20 dpi. In contrast, Uriarra was first detected in the contralateral lymph node at 6 dpi, and titres peaked at  $10^4$  pfu/g at 10 dpi (Figure 5.1C). Uriarra was not detected at 15 and 20 dpi in domestic rabbits.

Virus was not detected in the testes of TKO-infected domestic rabbits at 2, 4, 6, 10 or 20 dpi (data not shown).

### **5.2.1.2 Tissue damage and histopathological changes in the skin and draining lymph node of TKO-vaccinated domestic rabbits**

#### *5.2.1.2.1 Histological appearance of normal rabbit skin*

Haematoxylin and eosin-stained sections of skin from the dorsum of the hind foot on an uninfected rabbit are shown in Figure 5.2. The skin of uninfected rabbits has a thin epidermis consisting of a layer of cells with flattened nuclei covered with keratin (Figure 5.2A). The dermis consists of regularly packed collagen fibres and contains hair follicles and blood vessels (Figure 5.2). Within the dermis, many cell types can be differentiated based on their morphology and appearance after haematoxylin and eosin staining. The most common cell type in normal skin are fibroblasts (elongated darkly-stained cells; Figure 5.2B). Lymphocytes (10  $\mu$ M round cells with very little cytoplasm) and polymorphonuclear cells (PMN; 15-20  $\mu$ M, multilobed nuclei with dark pink-staining granular cytoplasm) are seen in infected skin (Figure 5.3), but these cell types are not common in uninfected skin. The total thickness of uninfected skin on the dorsum of the hind foot was between 1.5 and 2 mm on domestic rabbits.



**Figure 5.2** - Haematoxylin and eosin-stained skin section from the dorsum of the hind foot of uninfected domestic rabbits showing normal skin structure and cell types. **A:** The epidermis and upper dermis. **B:** The deeper dermis; blood vessels are surrounded by a layer of endothelial cells (arrowed). Bars on images are 50  $\mu\text{m}$  in length. Key: E, epidermis; D, dermis; HF, hair follicle; BV, blood vessel; F, fibroblast. Examples of each cell type and structure are indicated.

#### 5.2.1.2.2 Histopathological changes in the skin at the inoculation site of domestic rabbits following TKO infection

Haematoxylin and eosin-stained sections of skin at the inoculation site from three TKO-infected domestic rabbits at each time point were examined. Sections from Uriarra-infected rabbits prepared in a previous study (Best *et al.*, 2000; Best and Kerr, 2000) were re-examined and compared. The histological changes seen in skin at the inoculation site of TKO- and Uriarra-infected domestic rabbits are summarised in Table 5.1. Figures 5.3 to 5.6 show typical histopathology sections illustrating the changes.

At 2 dpi, there were no histological changes in the skin of TKO-infected rabbits (Figure 5.3A). By 4 dpi, the epidermis of TKO-infected rabbits was 1.5 times thicker than normal (Figure 5.3B). An increased cellular infiltrate was seen throughout the dermis, consisting of polymorphonuclear cells with some lymphocytes and other mononuclear cells (Figure 5.3B and C). Degradation of the regular collagen structure and purple-staining fibrin deposits was seen in the deeper dermis (Figure 5.3C).

By 6 dpi, the epidermis of TKO-infected rabbits at the inoculation site was 2-4 times thicker than normal (Figure 5.4A). There were more inflammatory cells in the upper dermis than at 4 dpi, consisting of mononuclear cells and polymorphonuclear cells (Figure 5.4). Large elongated cells previously identified as myxoma cells (Best *et al.*, 2000; Hurst, 1937) were seen in the deeper dermis of TKO-infected rabbits (Figures 5.4B), and were more common in Uriarra-infected rabbits (Table 5.1).

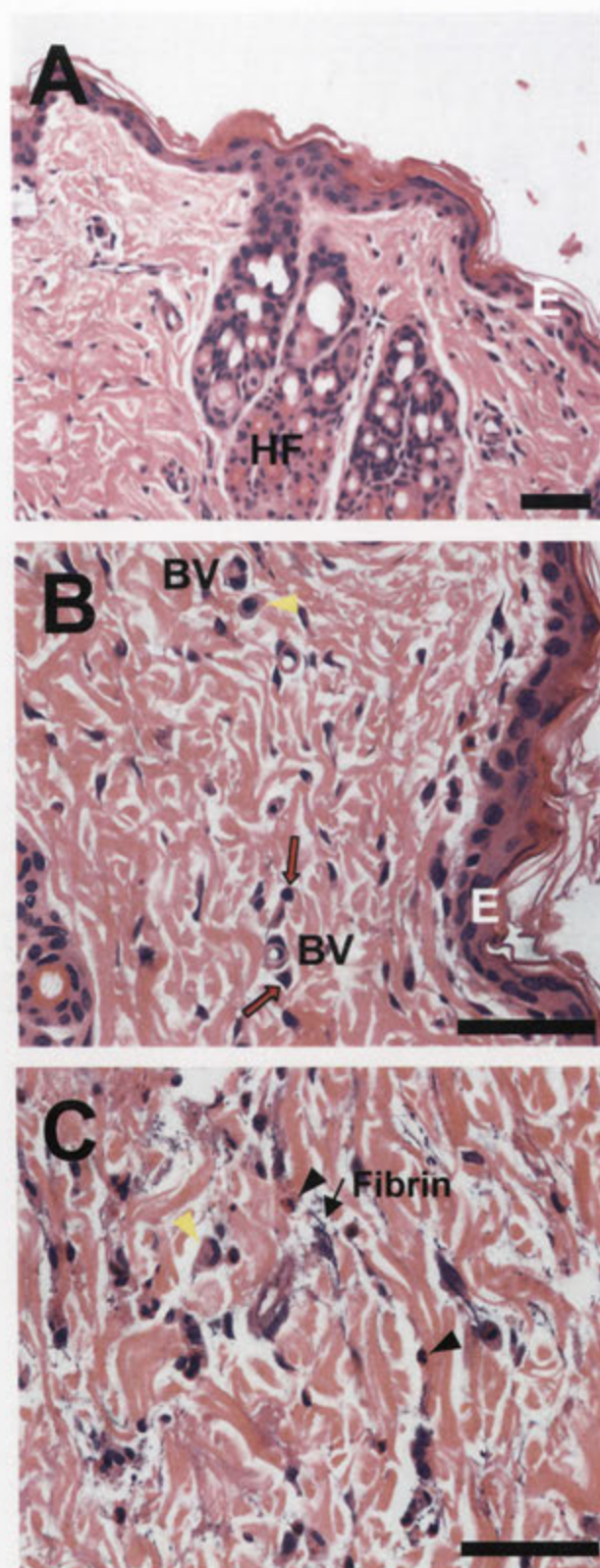
By 10 dpi, the primary lesions of TKO-infected rabbits were 4-6 mm thick and approximately 20 mm in diameter. Uriarra-induced lesions were up to 60 mm in diameter

**Table 5.1 - Summary of the histopathological changes in domestic rabbit skin following infection with TKO or Uriarra.** Histopathological images corresponding to the information in this table are found in Figures 5.3-5.6.

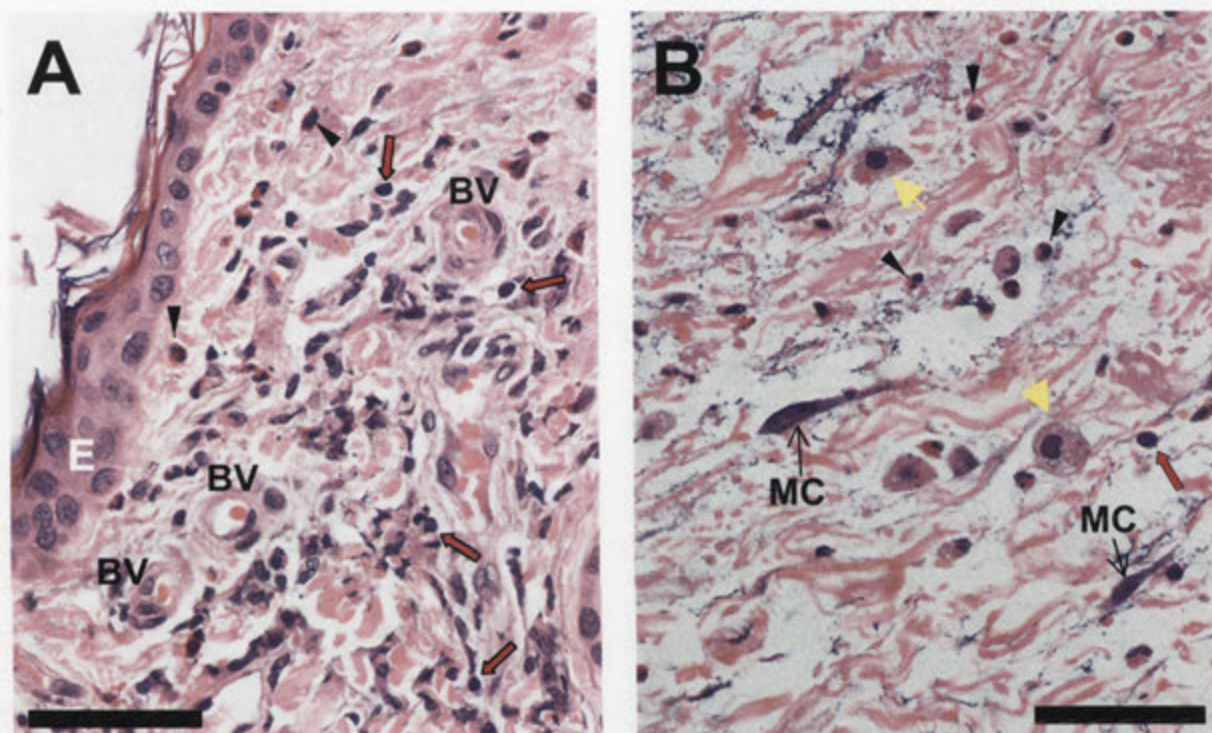
TKO		Uriarra
Two Days Post Infection		
No changes		No changes
Four Days Post Infection		
Epidermis	1.5 times thicker than normal; swelling of epidermal cells	1.5 times thicker than normal; swelling of epidermal cells
Dermis	Degradation of collagen fibres; fibrin deposition	Degradation of collagen fibres
Inflammation	Increased numbers of PMN near epidermis; mononuclear cells and PMN in dermis	Increased numbers of PMN in dermis
Six Days Post Infection		
Epidermis	2-4 times thicker than normal; some proliferation of epidermal cells.	3-5 times thicker than normal; proliferation of epidermal cells
Dermis	Oedema (skin section 2 times normal thickness); increased levels of collagen degradation and fibrin deposition than at 4 dpi; some myxoma cells in deeper dermis	Oedema (skin section 2 times normal thickness); degradation of collagen fibres throughout dermis; fibrin deposition; swelling of blood vessels; myxoma cells prevalent in deeper dermis
Inflammation	Cellular infiltrate consists of equal numbers of PMN and mononuclear cells; macrophages in deeper dermis; many more inflammatory cells than at 4 dpi	PMN infiltrate; occasional mononuclear cells
Ten Days Post Infection		
Epidermis	2 rabbits - thickening of epidermis 3-5 times normal; slight swelling of epidermal cells 1 rabbit - scabbing and complete loss of epidermal cell layer,	5 times thicker than normal; proliferation and degradation of cells
Dermis	Oedema (skin section 3-4 times normal thickness); extensive degradation of collagen fibres, especially near epidermis; proteinaceous exudate in upper dermis; no myxoma cells	Oedema (skin section 3-4 times normal thickness); extensive degradation of collagen fibres and fibrin deposits throughout; myxoma cells in deeper dermis
Inflammation	Very intense mononuclear cell infiltrate throughout the dermis, dominated by lymphocytes. No PMN present.	Infiltrate was predominantly PMNs; macrophages and mononuclear cells more common than at 6 dpi
Twenty Days Post Infection		
Epidermis	Normal	Very thick scab over the primary lesion; adjacent epidermis up to 10 times normal thickness; cellular proliferation
Dermis	Almost returned to normal; slightly irregular collagen structure	Oedema (skin section 3-5 times normal thickness); highly degraded dermis; vacuoles in dermis with extensive fibrin deposits
Inflammation	Slight mononuclear cell infiltrate, mostly lymphocytes	Intense mononuclear cell infiltrate throughout dermis; some PMN present

Abbreviations: PMN; polymorphonuclear cell; dpi, days post infection.





**Figure 5.3 - Histopathology of the skin at the inoculation site of domestic rabbits infected with TKO.** **A:** Skin at 2 days post infection. **B:** The epidermis and upper dermis at 4 days post infection. **C:** Inflammatory cell infiltrate, collagen degradation and fibrin deposits in the deeper dermis at 4 days post infection. Bars on images are 50 μm in length. Key: E, epidermis; BV, blood vessel; black arrowhead, polymorphonuclear cell; yellow arrowhead, macrophage; red arrow, lymphocyte. Examples of each cell type and structure are indicated



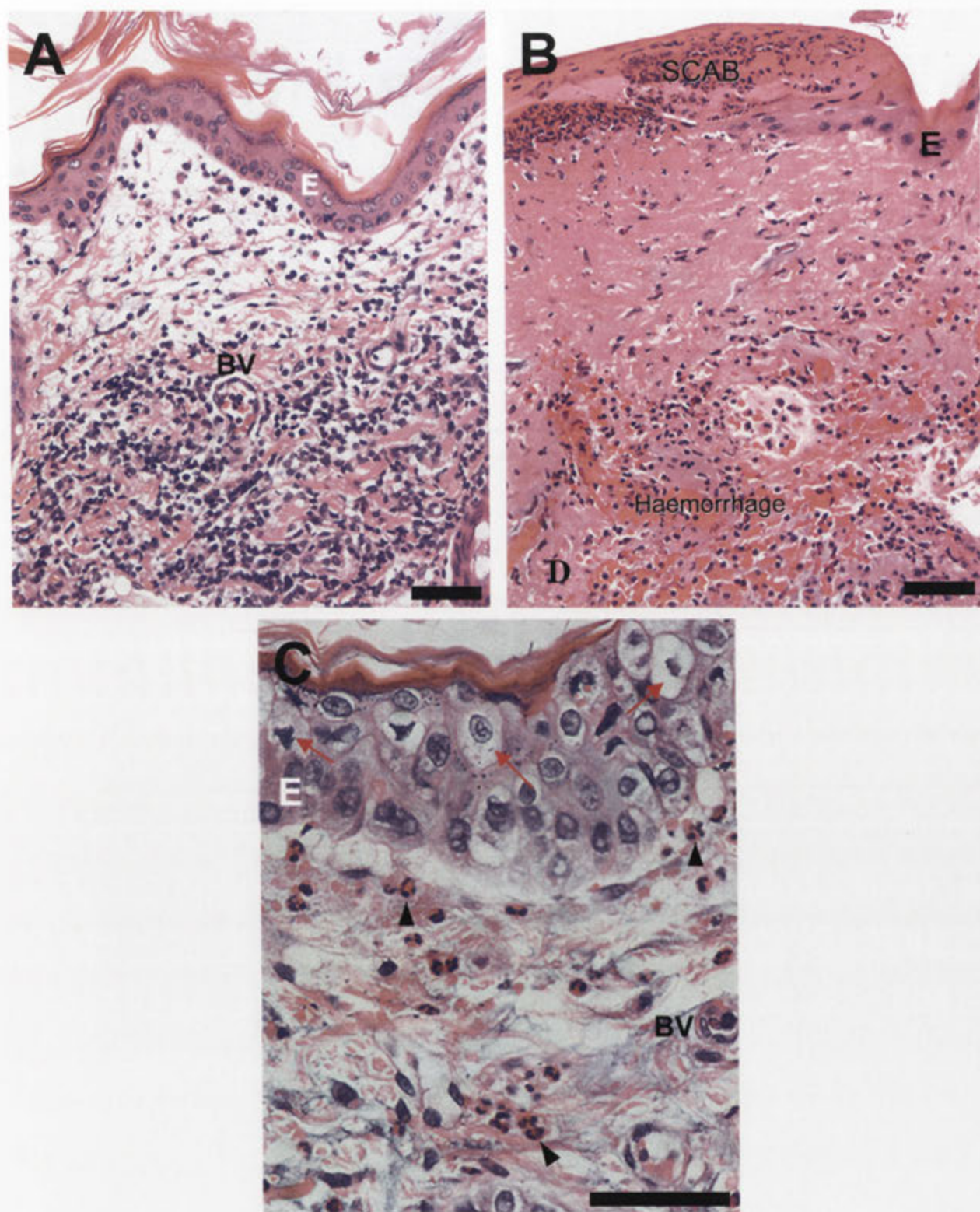
**Figure 5.4 - Histopathology of the skin at the inoculation site of domestic rabbits 6 days after infection with TKO.** **A:** Epidermis and upper dermis with an infiltrate of polymorphonuclear and mononuclear cells. **B:** Extensive collagen degradation, fibrin deposits, myxoma cells and an infiltrate of polymorphonuclear and mononuclear cells in the deeper dermis. Bars on images are 50  $\mu$ m in length. Key: E, epidermis; BV, blood vessel; black arrowhead, polymorphonuclear cell; yellow arrowhead, macrophage; red arrow, lymphocyte; MC, myxoma cell. Examples of each cell type and structure are indicated.

at this time (Best and Kerr, 2000). The epidermis of two TKO-infected rabbits was only slightly thicker than at 6 dpi, and the structure of the upper dermis was highly degraded and separated from the epidermis (Figure 5.5A). The third animal had a thick scab over the inoculation site and the dermis was filled with a dark pink-staining material and red blood cells (Figure 5.5B). The inflammatory infiltrate in TKO-infected rabbit skin consisted of mononuclear cells, predominantly lymphocytes (Figure 5.5A), and was more intense than at 6 dpi (Figure 5.4). In contrast, polymorphonuclear cells dominated the cellular infiltrate in Uriarra-infected skin at 10 dpi (Figure 5.5C). Also, the epidermis was 50% thicker than in TKO-infected rabbits, and contained more numerous degenerating epidermal cells (Figure 5.5C).

By 20 dpi, the skin of TKO-inoculated domestic rabbits had returned to normal appearance, although the collagen structure was still slightly irregular (Figure 5.6A) compared to uninfected controls (Figure 5.2). In contrast, Uriarra-infected skin was still very abnormal, with a very thick epidermis and an extensive inflammatory infiltrate consisting predominantly of macrophages and lymphocytes, although numerous polymorphonuclear cells were also present (Figure 5.6B).

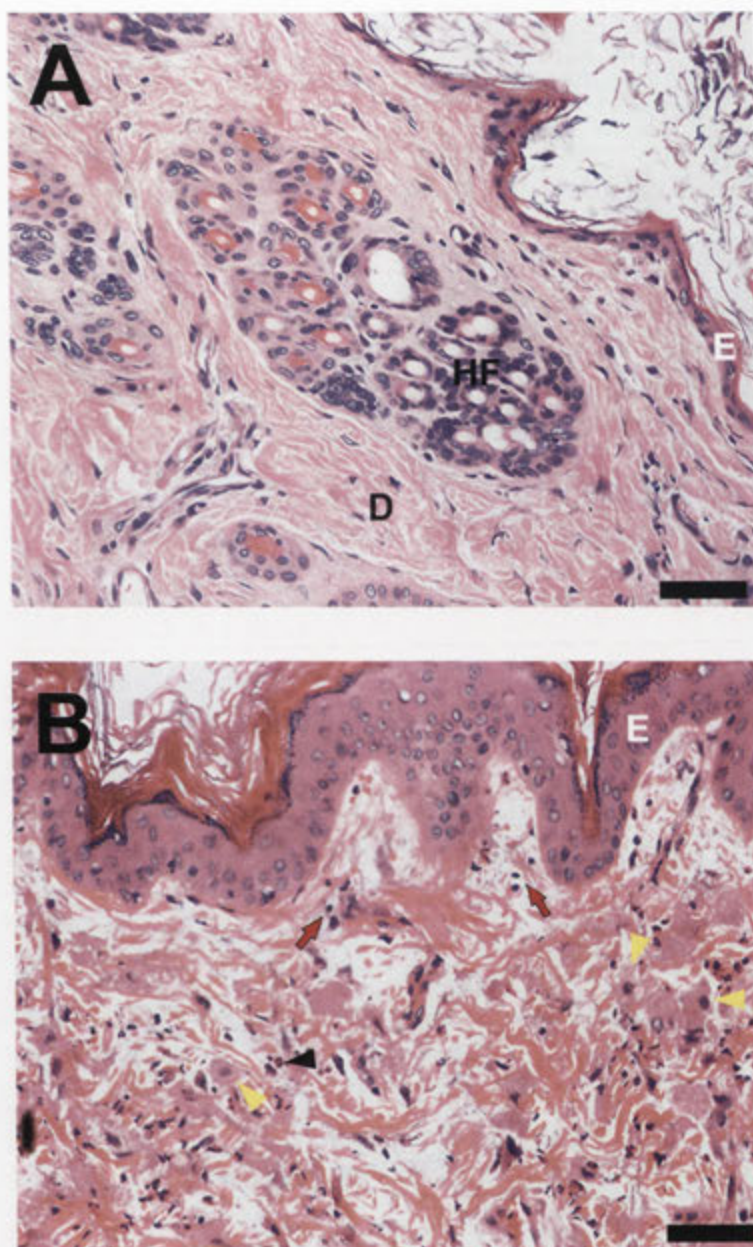
In summary, the histopathological changes that occurred in rabbit skin following TKO infection were the thickening of the epidermis and some proliferation of epidermal cells; swelling and degradation of the dermis; the infiltration of polymorphonuclear cells between 4 and 6 dpi, followed by an extensive influx of mononuclear cells including numerous lymphocytes at 10 dpi. By 20 dpi, the skin at the inoculation site had returned to normal structure and appearance. Uriarra-infected skin had a similar appearance to TKO-infected skin at 2, 4 and 6 dpi. However, at 10 dpi, the inflammatory infiltrate consisted





**Figure 5.5 - Histopathology of the skin at the inoculation site of domestic rabbits 10 days after infection with myxoma virus.** Skin sections from TKO-infected rabbits (A and B). Skin section from a Uriarra-infected rabbit (C). **A:** Skin of a TKO-infected rabbit with an extensive mononuclear cell infiltrate in the dermis. **B:** Skin of a TKO-infected rabbit showing scabbing, haemorrhage and pink-staining proteinaceous exudate. **C:** Epidermis of a Uriarra-infected rabbit showing degrading cells (thin red arrows) and polymorphonuclear cells in the dermis. Bars on images are 50 µm in length. Key: E, epidermis; D, dermis; BV, blood vessel; black arrowhead, polymorphonuclear cell. Examples of each cell type and structure are indicated.





**Figure 5.6 - Histopathology of the skin at the inoculation site of domestic rabbits 20 days infection with myxoma virus.** **A:** The epidermis and upper dermis of a TKO-infected rabbit. **B:** The epidermis and upper dermis of a Uriarra-infected rabbit. Bars on images are 50 µm in length. Key: E, epidermis; D dermis; HF, hair follicle; red arrow, lymphocyte; yellow arrowhead, macrophage; black arrowhead, polymorphonuclear cell. Examples of each cell type and structure are indicated.

predominantly of polymorphonuclear cells, and the skin was still abnormal in structure at 20 dpi.

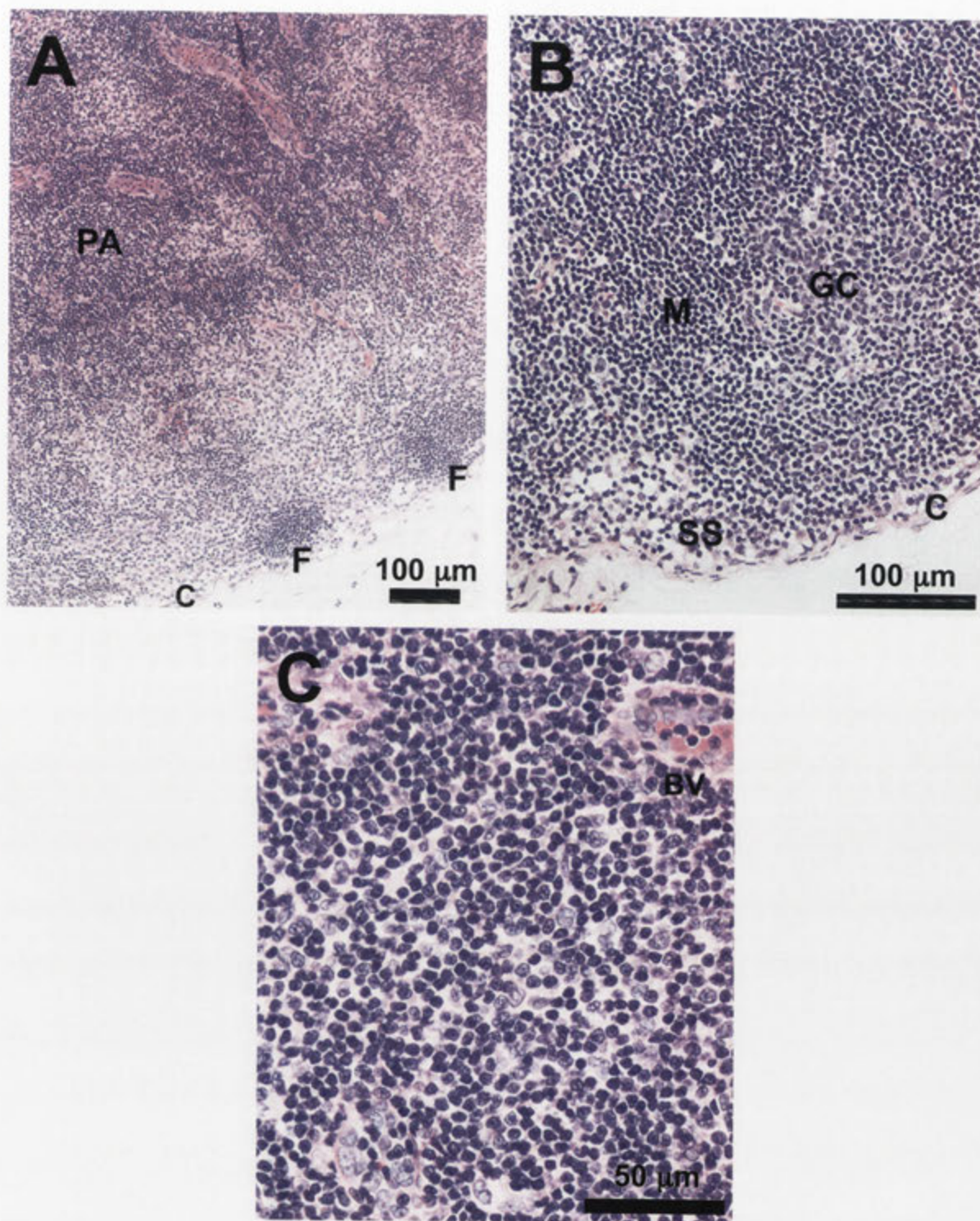
#### *5.2.1.2.3 Histological appearance of normal rabbit popliteal lymph node*

Images of haematoxylin and eosin-stained uninfected rabbit popliteal lymph nodes are shown in Figure 5.7. The lymph node is encased within a capsule of dense connective tissue (Figure 5.7A and B). Uninfected lymph nodes contain large numbers of primary lymphoid follicles with small lymphocytes immediately beneath the capsule (Figure 5.7A). Secondary lymphoid follicles, consisting of a germinal centre surrounded by a mantle of lymphocytes, are occasionally seen in uninfected lymph nodes (Figure 5.7B). A germinal centre is an active B cell zone where naive B cells are activated and differentiate into plasma cells (MacLennan, 1994). Active germinal centres have fewer lymphocytes than the surrounding mantle, and therefore stain more faintly, as shown in Figure 5.7B. The paracortical area is located beneath the follicle-containing cortex (Figure 5.7A). This region contained large numbers of lymphocytes and the occasional blood vessel (Roitt, 1997; Figure 5.7C).

#### *5.2.1.2.4 Histopathological changes in the draining lymph node of domestic rabbits following TKO infection*

Haematoxylin and eosin-stained sections of the draining lymph node from three TKO-infected domestic rabbits at each time point were examined. Sections from Uriarra-infected rabbits prepared in a previous study (Best *et al.*, 2000; Best and Kerr, 2000) were re-examined and compared. The histological changes seen in the draining lymph node of TKO- and Uriarra-infected rabbits are summarised in Table 5.2. Figures 5.8 to 5.10 show typical histopathology sections illustrating the changes.





**Figure 5.7 - Histology of the popliteal lymph node from an uninfected rabbit.** **A:** The cortex of the lymph node with primary lymphoid follicles and the paracortical area. **B:** Secondary lymphoid follicle with a germinal centre surrounded by the lymphocyte mantle. **C:** The paracortical area with abundant lymphocytes. Key: GC, germinal centre; M, mantle; F, primary lymphoid follicle; C, capsule; PA, paracortical area; SS, subcapsular sinus; BV, blood vessel. Examples of each cell type and structure are indicated.

**Table 5.2 - A summary of the histopathological changes in domestic rabbit draining lymph nodes following infection with TKO or Uriarra.** Histopathological images corresponding to the information in this table are found in Figures 5.8-5.10.

TKO		Uriarra
Two Days Post Infection		
Cortex	Large secondary lymphoid follicles with distinct germinal centres; macrophages in mantle	Large secondary lymphoid follicles, no distinct germinal centres; macrophages throughout secondary lymphoid follicles
Cellular infiltrate	PMN and eosinophilic mononuclear cells in the medullary sinus and cortex	PMN and eosinophilic mononuclear cells in the medullary sinus
Paracortex	Normal	Normal
Four Days Post Infection		
Cortex	Increase in number of active germinal centres since 2 dpi	Large increase in number of active germinal centres since 2 dpi, found into paracortex
Cellular infiltrate	As at 4 dpi; PMN and eosinophilic mononuclear cells clustering in medullary sinus	No change
Paracortex	Normal	Normal
Six Days Post Infection		
Cortex	Numerous large active germinal centres	Fewer, smaller germinal centres than in TKO-infected animals; large parts of cortex highly depleted of lymphocytes with extensive apoptosis
Cellular infiltrate	No change	Very intense PMN infiltrate throughout node, especially in medullary sinus
Paracortex	Slight, localised depletion of lymphocytes compared to uninfected lymph nodes	Very low density of lymphocytes; extensive apoptosis visible
Ten Days Post Infection		
Cortex	Numerous large germinal centres containing mitotic and apoptotic cells; slightly depleted of lymphocytes; haemorrhaging in the cortex	Fewer germinal centres than in TKO-infected rabbits; PMN and apoptotic cells present in germinal centres
Cellular infiltrate	Fewer PMN seen in lymph node; macrophages containing apoptotic bodies seen in germinal centres	Fewer PMN in lymph node; some macrophages containing apoptotic bodies seen in germinal centres
Paracortex	Slightly lower density of lymphocytes compared to uninfected lymph nodes; clustering of lymphocytes in blood vessels	Low density of lymphocytes, though not as marked as at 6 dpi; no apoptosis visible microscopically
Twenty Days Post Infection		
Cortex	Numerous large active germinal centres in cortex	Few germinal centres; cortex depleted of lymphocytes
Cellular infiltrate	Normal	Large numbers of macrophages seen in all parts of the lymph node
Paracortex	Normal	Very low density of lymphocytes; remaining lymphocytes were clustered around macrophages

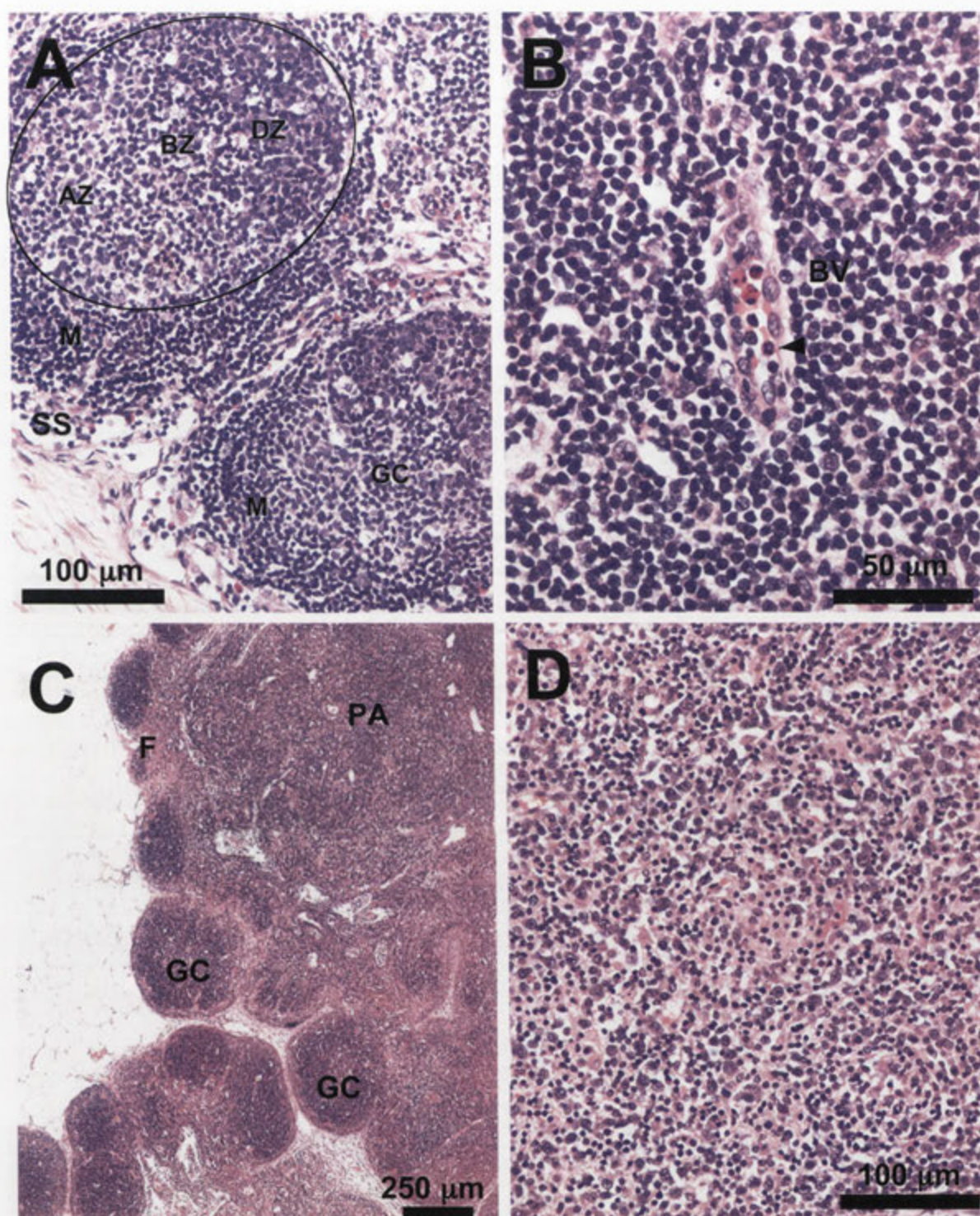
Abbreviations: PMN, polymorphonuclear cells; dpi, days post infection.



At 2 dpi, secondary lymphoid follicles with lighter-staining germinal centres were prevalent in the draining lymph nodes of TKO-infected animals. Secondary lymphoid follicles were also seen in lymph nodes of Uriarra-infected rabbits, but lighter staining germinal centres indicative of active B cell zones (MacLennan, 1994) were not seen. By 4 dpi, germinal centres were prevalent in the draining lymph nodes of both TKO (Figure 5.8A) and Uriarra-infected rabbits. Many of these germinal centres had developed dark zones, basal light zones and apical light zones typical of active germinal centres (Roitt, 1997; Figure 5.8A).

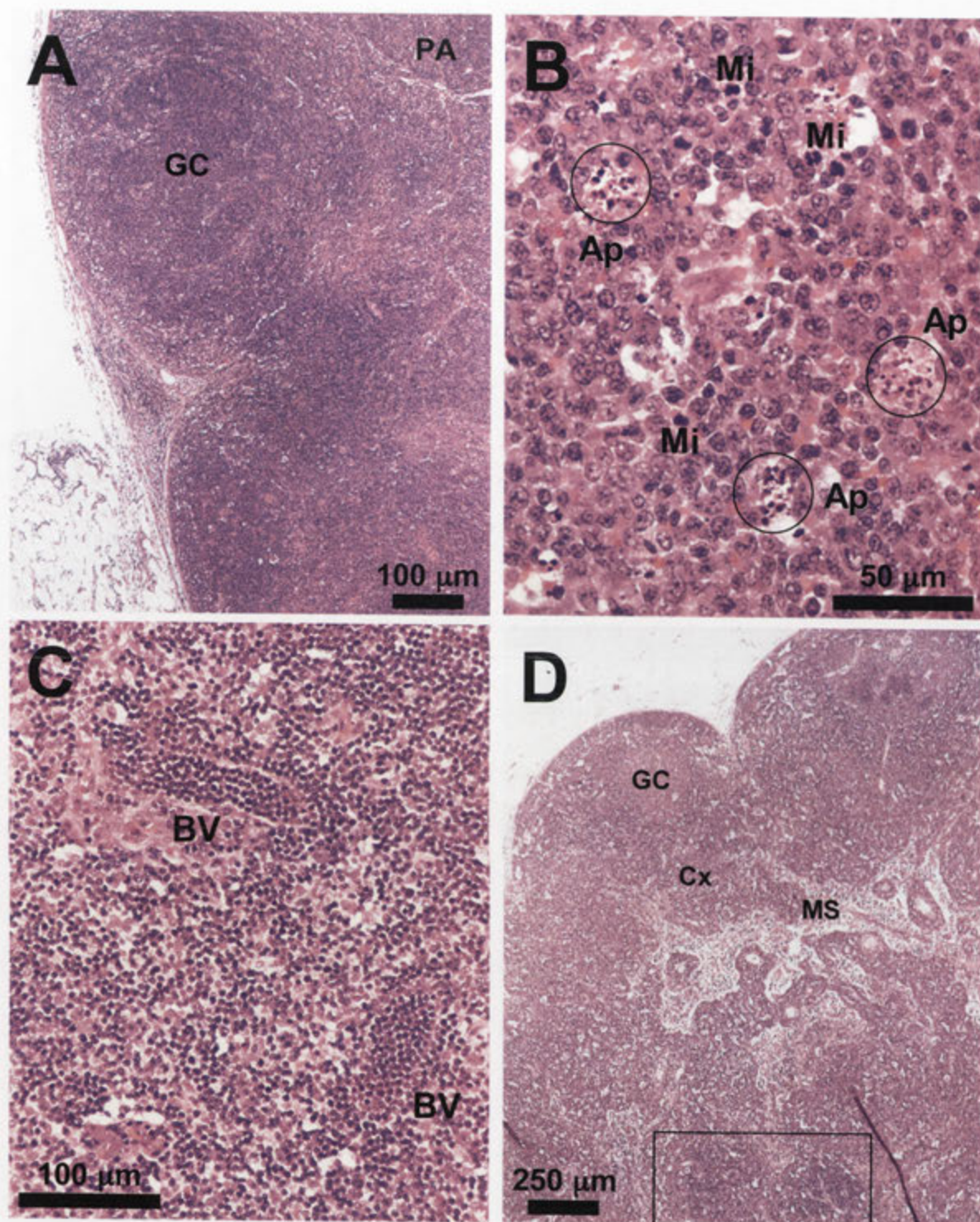
At 6 dpi, the draining lymph nodes of TKO-infected domestic rabbits had swollen to 2.5-3.5 times normal size with a blotchy appearance and some haemorrhagic patches. Numerous active germinal centres were present (Figure 5.8C). The paracortical area had a reduced lymphocyte density in some areas (Figure 5.8D) compared to normal lymph nodes (Figure 5.7C), although there was no evidence of lymphocyte apoptosis. Most of the paracortex was still densely populated with lymphocytes. In contrast, the draining lymph nodes of Uriarra-infected animals were reported to be sparsely populated with lymphocytes throughout most of the node, with extensive apoptosis evident throughout the cortex and paracortex (Best *et al.*, 2000). Other regions of the cortex of these lymph nodes were still well populated with lymphocytes, with active germinal centres and densely populated mantles.

At 10 dpi, the draining lymph nodes of TKO-infected rabbits were still 2.5-3 times normal size with a blotchy appearance and haemorrhagic patches. The germinal centres contained more lymphocytes than at 6 dpi, and both apoptotic and mitotic cells were present (Figure 5.9B). There was haemorrhaging in the cortex in all three infected rabbits, indicated by erythrocytes outside of blood vessels (data not shown). Blood vessels within the paracortex



**Figure 5.8 - Histopathology of the draining popliteal lymph node from domestic rabbits after TKO infection.** Sections from TKO-infected rabbits at 4 dpi (A and B) and 6 dpi (C and D). **A:** Germinal centre in the lymph node at 4 dpi with distinct dark, basal light and apical light zones. **B:** Paracortex of the lymph node at 4 dpi. **C:** Germinal centres in the draining lymph node at 6 dpi. **D:** Paracortex of TKO-infected rabbit at 6 dpi was depleted of lymphocytes in some areas. Key: GC, germinal centre; M, mantle; F, primary lymphoid follicle; PA, paracortical area; SS, subcapsular sinus; BV, blood vessel; DZ, dark zone; BZ, basal light zone; AZ, apical light zone; black arrowhead, polymorphonuclear cell. Examples of each cell type and structure are indicated.





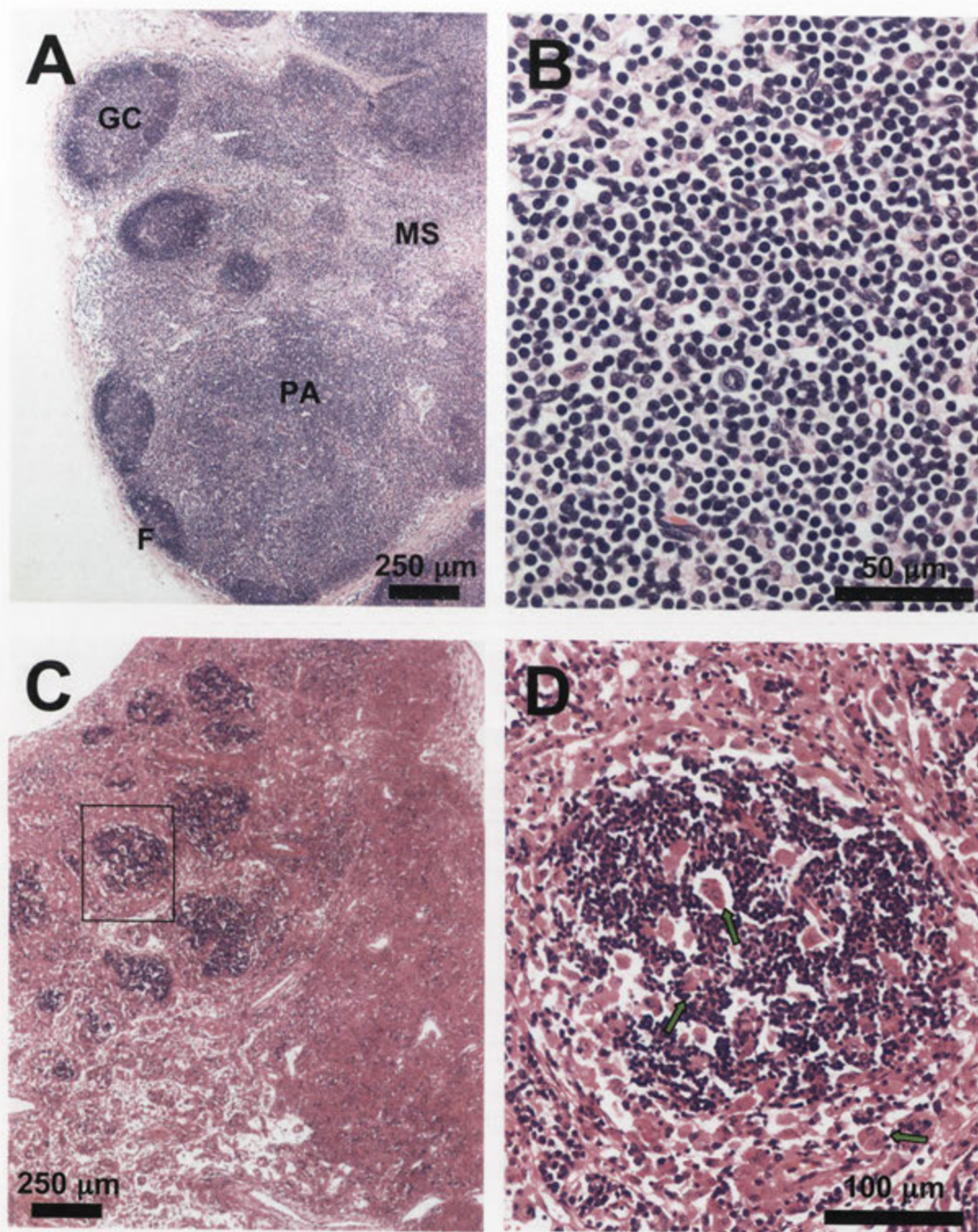
**Figure 5.9 - Histopathology of the draining popliteal lymph node from domestic rabbits 10 days after myxoma virus infection.** Sections from TKO-infected rabbits (A-C). Section from a Uriarra-infected rabbit (D). **A:** Draining lymph node of a TKO-infected rabbit. **B:** Higher magnification image of a germinal centre in the lymph node of a TKO-infected rabbit with apoptotic and mitotic cells. **C:** The paracortex of a TKO-infected rabbit with a reduced density of lymphocytes and with lymphocytes clustered in blood vessels. **D:** Draining lymph node of a Uriarra-infected rabbit was depleted of lymphocytes as indicated by the lack of dark purple staining compared to a TKO-infected rabbit (A). Some areas had darker purple staining (box). Key: GC, germinal centre; Cx, cortex; PA, paracortical area; MS, medullary sinus; BV, blood vessel; Ap, apoptotic bodies; Mi, mitotic cell. Examples of each cell type and structure are indicated.

were densely packed with lymphocytes (Figure 5.9C). Lymph nodes of Uriarra-infected domestic rabbits were less densely populated with lymphocytes (Figure 5.9D) compared to TKO-infected rabbits (Figure 5.9A) as seen by the light staining of sections.

At 20 dpi, the draining lymph nodes of TKO-infected rabbits had returned to a more normal appearance, with numerous active germinal centres (Figure 5.10A) which had clearly defined dark and light zones and densely populated mantles. The paracortical area was densely populated by lymphocytes (Figure 5.10B). In contrast, the lymph nodes of Uriarra-infected rabbits were sparsely populated with lymphocytes as seen by the lack of purple staining in lymph node sections (Figure 5.10C). The lymphocytes remaining in these lymph nodes were clustered around macrophage-like cells with extensive dendritic processes, some appearing to contain phagocytosed apoptotic bodies (Figure 5.10D).

In summary, following infection with TKO, the draining lymph nodes of domestic rabbits developed numerous secondary lymphoid follicles with active germinal centres by 2 dpi, there was a reduction in the density of lymphocytes in the cortex and paracortex at 6 and 10 dpi, but by 20 dpi, the draining lymph node appeared normal except for the presence of numerous large secondary lymphoid follicles. In contrast, the draining lymph nodes of Uriarra-infected rabbits did not form active germinal centres as rapidly as TKO-infected rabbits. At 6 dpi, extensive apoptosis was evident throughout lymph nodes. At 10 and 20 dpi, nodes were sparsely populated with lymphocytes compared to normal lymph nodes and those from TKO-infected rabbits.





**Figure 5.10** - Histopathology of the draining popliteal lymph node from domestic rabbits 20 days after myxoma virus infection. Sections from TKO-infected rabbits (A and B). Sections from Uriarra-infected rabbits (C and D). **A:** The draining lymph node of a TKO-infected rabbit appears similar to normal lymph node (Figure 5.7A) except for more numerous secondary lymphoid follicles. **B:** The paracortical area of the lymph node from a TKO-infected rabbit. **C:** Draining lymph node of a Uriarra-infected rabbit. **D:** Enlargement of the boxed area in C, showing a cluster of lymphocytes surrounding macrophage-like cells. Key: GC, germinal centre; F, primary lymphoid follicle; PA, paracortical area; MS, medullary sinus; green arrow, macrophage-like cell. Examples of each cell type and structure are indicated.

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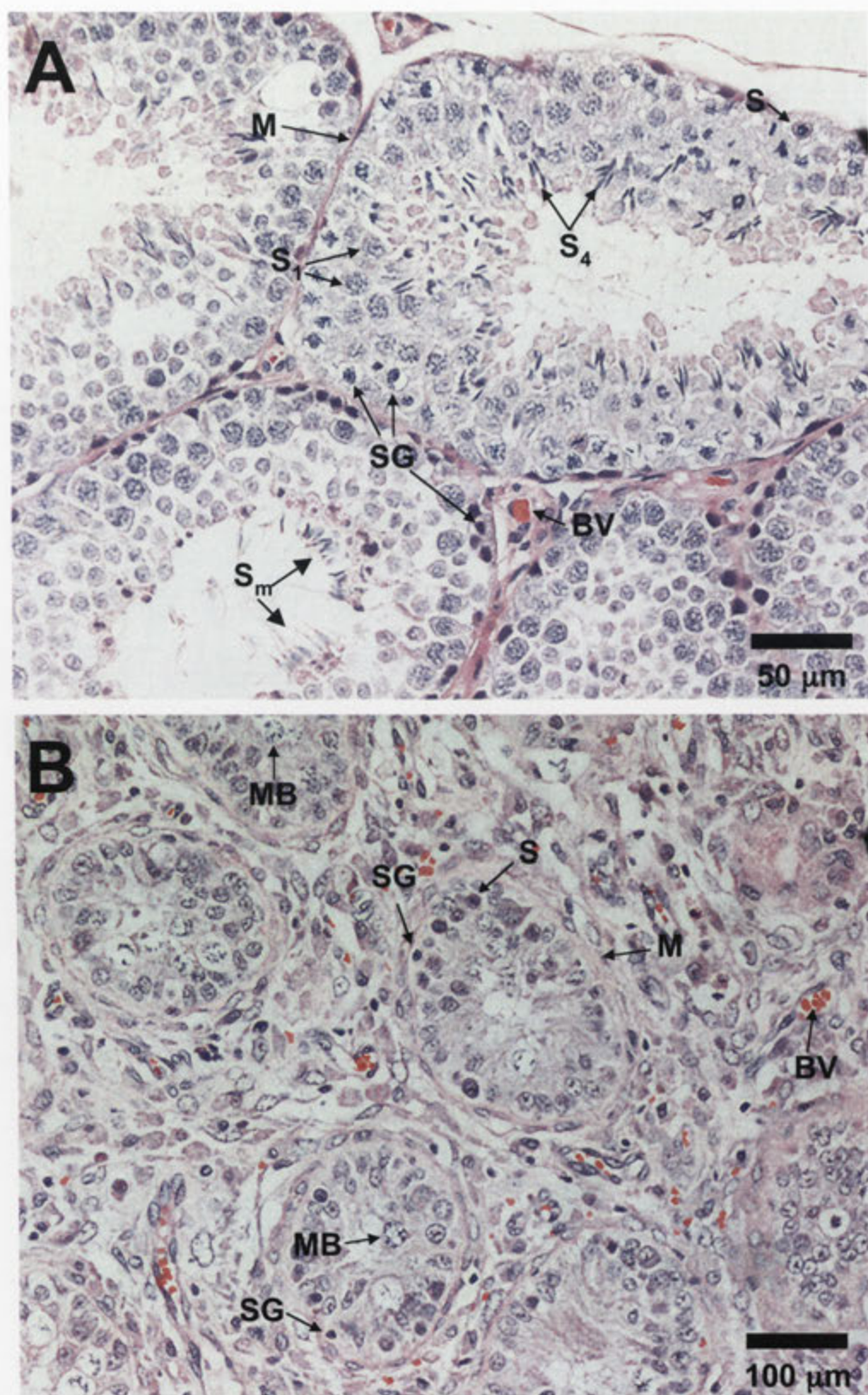
#### 5.2.1.2.5 Histological analysis of testes from TKO-infected domestic rabbits

The development of spermatozoa in the seminiferous tubules of the testis proceeds through a series of stages, which are identified in a section of a normal rabbit testis in Figure 5.11A. Development occurs in waves through each tubule, and early and late stages of development are generally not evident in all tubules (Guraya, 1987). Spermatogonia are identified as small cells with dense chromatin and a large nuclear vacuole (Figure 5.11A, SG). These enter the first stages of meiosis and change morphology into primary spermatocytes, distinguished as cells with coarser chromatin (Figure 5.11A, S<sub>1</sub>). The process of meiosis proceeds through several short-lived stages until elongated developing spermatozoa with darkly staining purple nuclei are formed (Figure 5.11A, S<sub>4</sub>), and then into mature tailed spermatozoa with broad, pale blue staining nuclei (Figure 5.11A; S<sub>m</sub>). Developing and tailed spermatozoa were rarely observed in the same tubule. Sertoli cells support the developing spermatozoa (Guraya, 1987; Wheeler *et al.*, 1987).

For comparison, a histological section of testis from a Uriarra-infected rabbit 20 days after inoculation is shown in Figure 5.11B. The degeneration of the seminiferous tubules is evident by their reduced size compared to normal testis and the absence of developmental stages of spermatozoa, with only spermatogonia and Sertoli cells evident. No mature or developing spermatozoa were found in any tubule from these rabbits. The inflammation of the testis is evident by the swelling of the interstitium (the connective tissue between the tubules) compared to normal testis samples.

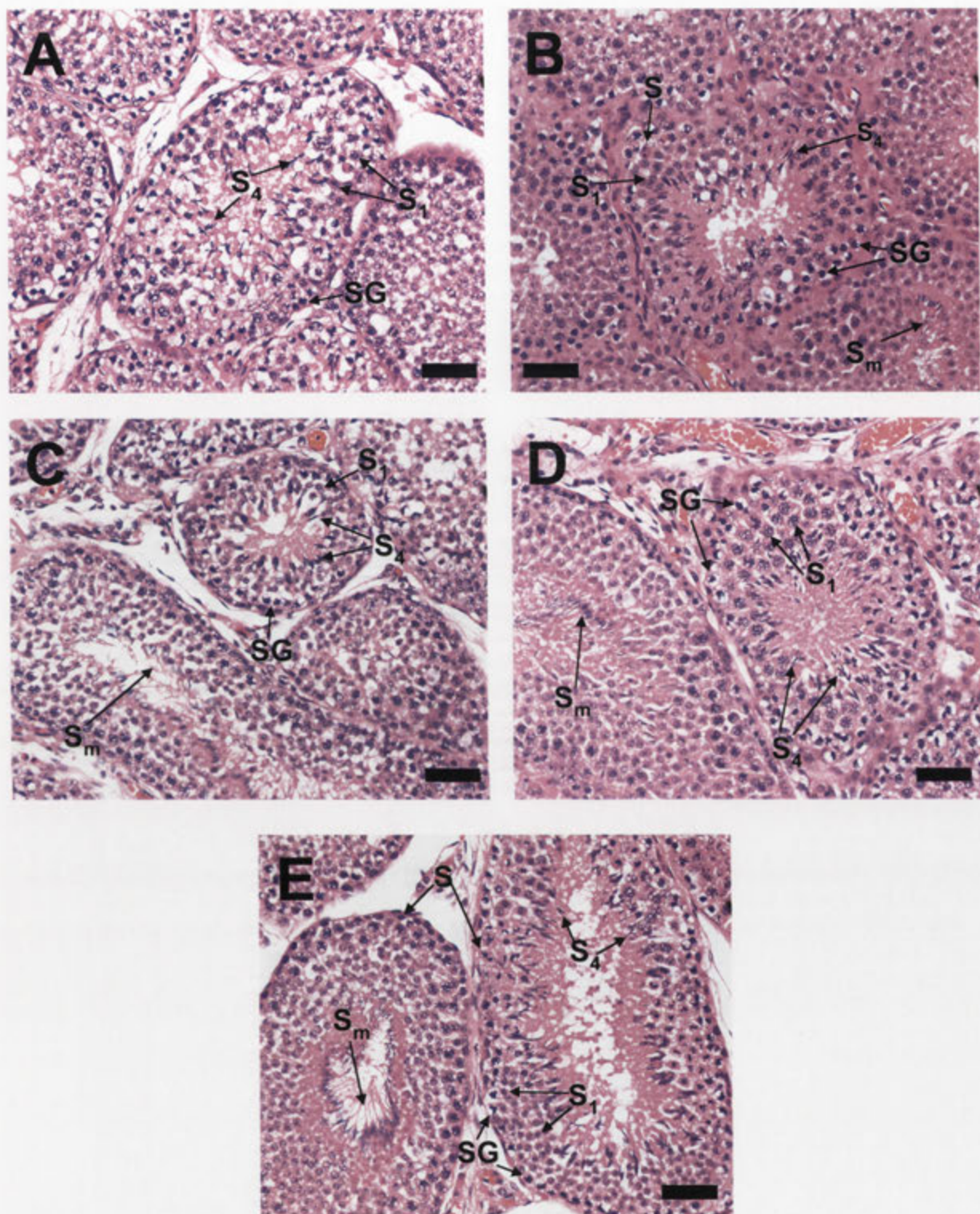
Histological analysis of testis samples from TKO-vaccinated rabbits revealed no significant abnormalities compared to normal testes at 2, 4, 6, 10 and 20 dpi. Figure 5.12 shows an example of seminiferous tubules from TKO-infected rabbits at each time after inoculation.





**Figure 5.11 - Histology of seminiferous tubules of domestic rabbits before and after Uriarra infection.** **A:** Tubules from an uninfected rabbit showing all stages of spermatozoa development. **B:** Tubules from a rabbit 20 days after Uriarra infection. Key: S, Sertoli cell; SG, spermatogonia; S<sub>1</sub>, primary spermatocytes; S<sub>4</sub>, elongated developing spermatozoa; S<sub>m</sub>, mature spermatozoa with tails; MB, multinucleate body; M, basement membrane; BV, blood vessel. Examples of each cell type and structure are indicated.





**Figure 5.12** - Seminiferous tubules of domestic rabbits following infection with TKO. Tubules from rabbits 2 days (A), 4 days (B), 6 days (C), 10 days (D) and 20 days (E) after infection with TKO are shown. Bars on images are 50  $\mu$ m in length. Key: S, Sertoli cell; SG, spermatogonia;  $S_1$ , primary spermatocytes;  $S_4$ , elongated developing spermatozoa;  $S_m$ , mature spermatozoa with tails. Examples of each cell type are indicated.



At all time points, the testes of TKO-infected rabbits displayed tubules with all stages of spermatozoa development evident, and were indistinguishable from uninfected rabbit samples. There was also no evidence of inflammation or other gross histopathological changes in the structure or appearance of the testis or epididymis.

## **5.2.2 Pathogenesis of TKO in mature wild rabbits**

The pathogenesis of TKO was examined in wild rabbits to determine the potential of the vaccine virus to be transmitted between wild rabbits and become established amongst wild rabbit populations in Australia. Rabbits were infected, tissues harvested, processed and analysed as described for domestic rabbits (Section 5.2.1). The titre of virus in the skin at the inoculation site, the draining popliteal lymph node and the contralateral lymph node were measured to examine the level of dissemination of the virus, and the potential transmissibility of the vaccine from an infected wild animal. The pathology of the skin at the inoculation site and the draining lymph node were also examined.

### *5.2.2.1 Titres of virus in wild rabbit tissues*

Figure 5.13 shows the virus titre for each wild rabbit in the skin at the inoculation site, the draining lymph node and the contralateral lymph node. Samples where a trace of virus (a single plaque on one of duplicate samples) was found were defined as having a titre of 50 pfu/g ( $10^{1.69}$ ), and samples with no detectable virus were defined as having a titre of 0 pfu/g. The mean titres of Uriarra determined by Best and Kerr (2000) have been included in Figure 5.13 to allow comparison of TKO to a field isolated virus that is readily transmissible between rabbits.

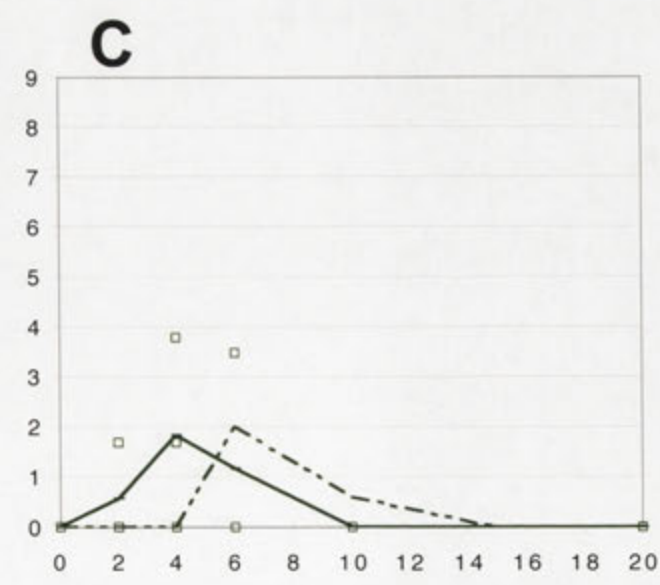
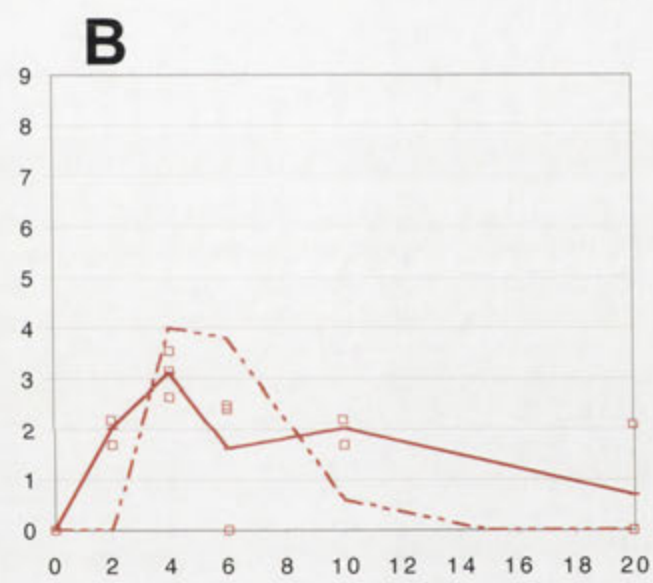
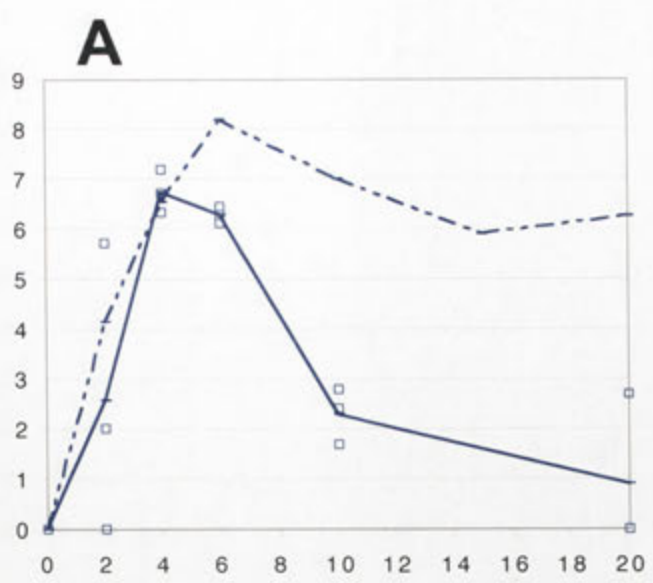
TKO was detected at the inoculation site of two out of three wild rabbits at 2 days post inoculation (dpi). By 4 dpi, virus titres were at their highest levels at a mean of  $5.5 \times 10^6$  pfu/g, with a range of  $2.2 \times 10^6$  to  $1.5 \times 10^7$  pfu/g (Figure 5.13A). By 6 dpi, virus titres in wild rabbits were consistently lower than those measured in domestic rabbits, with titres at a mean of  $1.9 \times 10^6$  pfu/g. Low titres of TKO were detected at the inoculation site of all three rabbits at 10 dpi (mean titre of 115 pfu/g) and in one of three rabbits 20 dpi at  $5 \times 10^2$  pfu/g. Uriarra titres were similar to TKO titres at 2 and 4 dpi, and peaked at over  $10^8$  pfu/g at 6 dpi (Best and Kerr, 2000). By 20 dpi, titres were still above  $10^6$  pfu/g (Figure 5.13A).

TKO titres in the draining lymph nodes of wild rabbits were similar to those in domestic rabbits. Virus was detected in all rabbits by 2 dpi and peaked at 4 dpi with titres between  $4.5 \times 10^2$  and  $3.6 \times 10^3$  pfu/g (Figure 5.13B). TKO was detected in two of three rabbits at 10 dpi with titres of  $1.5 \times 10^2$  pfu/g. At 20 dpi, TKO was detected ( $10^2$  pfu/g) in one rabbit (Figure 5.13B) which also had detectable virus at the inoculation site (Figure 5.13A). Uriarra was first detected in the draining lymph node at 4 dpi with a mean titre of  $10^4$  pfu/g. By 10 dpi, only a trace of virus was detected some wild rabbits, and Uriarra was not detected at 15 or 20 dpi (Figure 5.13B).

In the contralateral lymph nodes of wild rabbits, a trace of TKO was detected in one rabbit at 2 dpi and in two rabbits at 4 dpi (mean titre of 50 pfu/g; Figure 5.13C). At 6 dpi, TKO was detected in one rabbit at a titre of  $3 \times 10^3$  pfu/g, and was not detected at 10 or 20 dpi. Titres of Uriarra in the contralateral lymph node were similar to TKO, with a trace of virus measured in some rabbits at 6 and 10 dpi only (Figure 5.13C).

**Figure 5.13 - Myxoma virus titres in wild rabbit tissues following inoculation with 5000 pfu of TKO.** Virus titres at the inoculation site (A), draining lymph node (B) and contralateral lymph node (C) were measured by plaque assay, with a detection limit of  $10^2$  pfu/g. Each open square represents an individual rabbit infected with TKO and killed 2, 4, 6, 10 or 20 dpi. A titre of zero indicates no virus was detected in the given tissue sample. A titre of  $10^{1.69}$  pfu/g indicates that a single plaque (a trace) was detected on duplicate sample wells. The solid line on each graph represents the geometric mean titre for the three rabbits sampled at each time point. The dotted line represents the geometric mean titre of Uriarra (of 2 or 3 wild rabbits) in the same tissues following infection with 100 pfu of virus, as determined by Best and Kerr (2000).

Virus titre ( $\log_{10}$ [pfu/g tissue])



Time post infection (days)

#### 5.2.2.2 Histopathological changes in the skin of wild rabbits at the primary inoculation site following TKO infection

Haematoxylin and eosin-stained sections of the skin at the inoculation site from three TKO-infected wild rabbits at each time point were examined. Sections from Uriarra-infected wild rabbits prepared in a previous study (Best *et al.*, 2000; Best and Kerr, 2000) were re-examined and compared. The histological changes seen in the skin of TKO- and Uriarra-infected wild rabbits are summarised in Table 5.3. Figures 5.14 to 5.16 show typical histopathology sections illustrating the changes.

The changes observed during TKO or Uriarra infection in wild rabbit skin were similar to those seen in domestic rabbits, although there were several key differences. At 4 dpi, there was a more intense inflammatory cell infiltrate (Figure 5.14A) than seen in domestic rabbits at the same time (Figure 5.3B). At 6 dpi, the inflammatory cell infiltrate was much more intense than that in domestic rabbits at the same time and in two of the three rabbits was predominantly mononuclear cells, with polymorphonuclear cells almost absent (Figure 5.14C and D). This was similar to domestic rabbits at 10 dpi (Figure 5.5A). The pathology of one TKO-infected wild rabbit appeared similar to that of domestic rabbits at the same time point, with a predominantly polymorphonuclear cell infiltrate (Figure 5.14B).

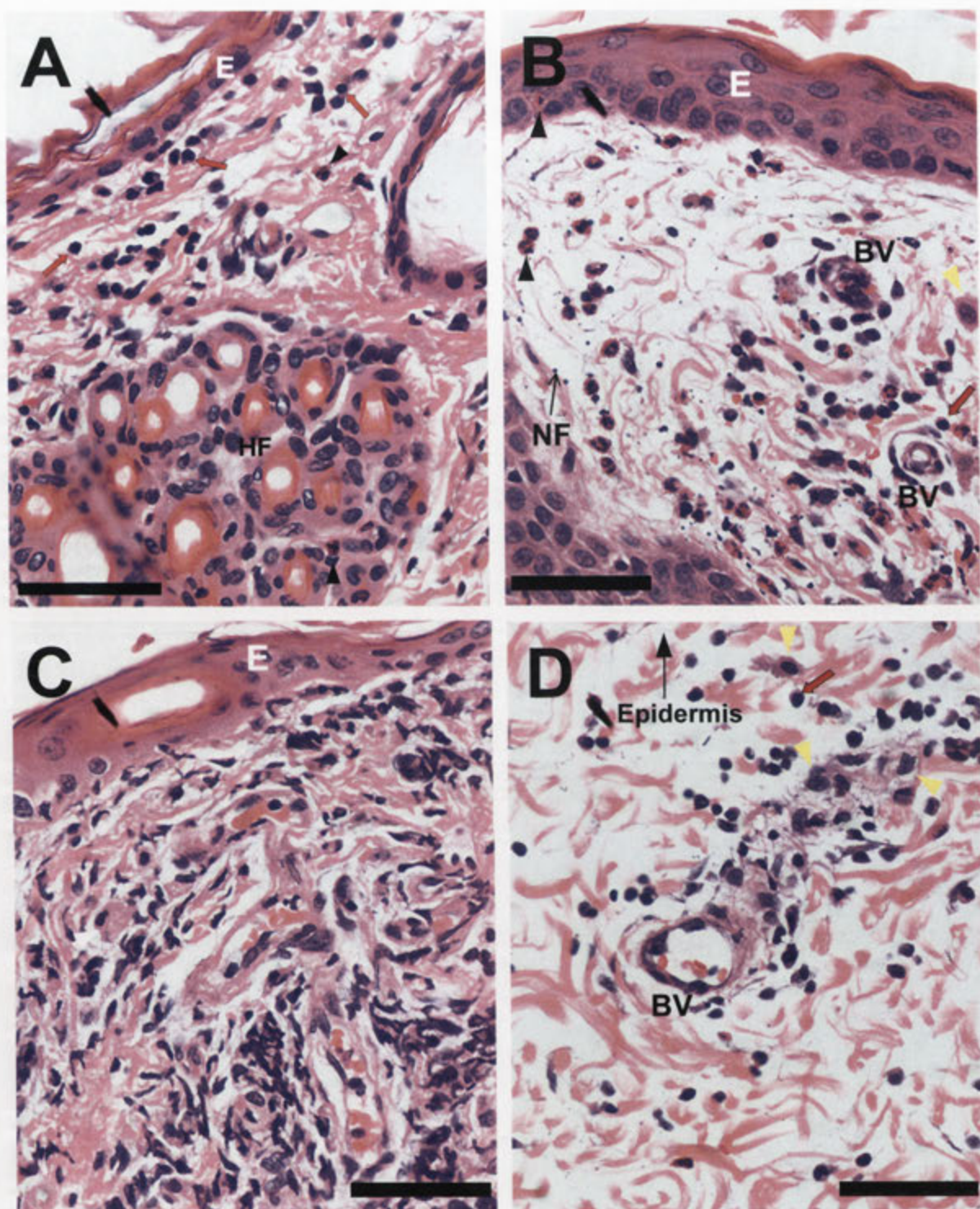
At 10 dpi, the inflammatory infiltrate in wild rabbit skin was similar to that in domestic rabbit skin at the same time, with an intense mononuclear cell infiltrate throughout the dermis (Figure 5.15A and B). The epidermis appeared normal, though darkly stained (Figure 5.15A) and the collagen structure of the deeper dermis was relatively intact (Figure 5.15B) compared to domestic animals (Figure 5.5A). In contrast, the dermis of Uriarra-infected wild rabbits was highly degraded, with almost no collagen in the upper dermis and

**Table 5.3 - A summary of the histopathological changes of wild rabbit skin following infection with TKO or Uriarra.** Histopathological images corresponding to the information in this table are found in Figures 5.14-5.16.

TKO		Uriarra
Two Days Post Infection		
	No change	No change
Four Days Post Infection		
Epidermis	No change	No change
Dermis	Degradation of collagen fibres; fibrin deposition; extensive haemorrhaging in one rabbit	Degradation of collagen fibres
Inflammation	Increased presence of PMN and mononuclear cells near epidermis and in dermis	Slight increase in PMN and mononuclear cells in dermis
Six Days Post Infection		
Epidermis	2-4 times thicker than normal; some proliferation of epidermal cells.	3-4 times thicker than normal; proliferation of epidermal cells
Dermis	Oedema (skin sections 3 times normal thickness); slightly increased levels of collagen degradation and fibrin deposition; no myxoma cells	Oedema (skin sections 3 times normal thickness); degradation of collagen fibres throughout dermis; fibrin deposition; swelling of blood vessels; myxoma cells in deeper dermis
Inflammation	Increased cellular infiltrate consisting of PMN and mononuclear cells; composition varied between animals	Mixed PMN and mononuclear cell infiltrate throughout dermis - much less than in TKO-infected animals
Ten Days Post Infection		
Epidermis	Normal	5 times thicker than normal; proliferation and degradation of cells
Dermis	Oedema (skin sections 4 times normal thickness); extensive degradation of collagen fibres near epidermis; deeper dermis only had slight degradation; no myxoma cells	Oedema (skin sections 5 times normal thickness); extensive degradation of collagen fibres, fibrin deposits throughout dermis; proteinaceous exudate in upper dermis; some myxoma cells in deeper dermis
Inflammation	Very intense mononuclear cell infiltrate throughout the dermis, dominated by lymphocytes and macrophages. No PMN present.	Infiltrate was predominantly mononuclear cells; PMN present throughout dermis
Twenty Days Post Infection		
Epidermis	Normal	Very thick scab over the primary lesion; adjacent epidermis up to 10x normal thickness; cellular proliferation
Dermis	Almost returned to normal; slightly irregular collagen structure	Oedema (skin sections 6 times normal thickness); highly degraded dermis; vacuoles in dermis with extensive fibrin deposits
Inflammation	Slight mononuclear infiltrate, mostly lymphocytes	Intense mononuclear cell infiltrate throughout dermis; some PMN present

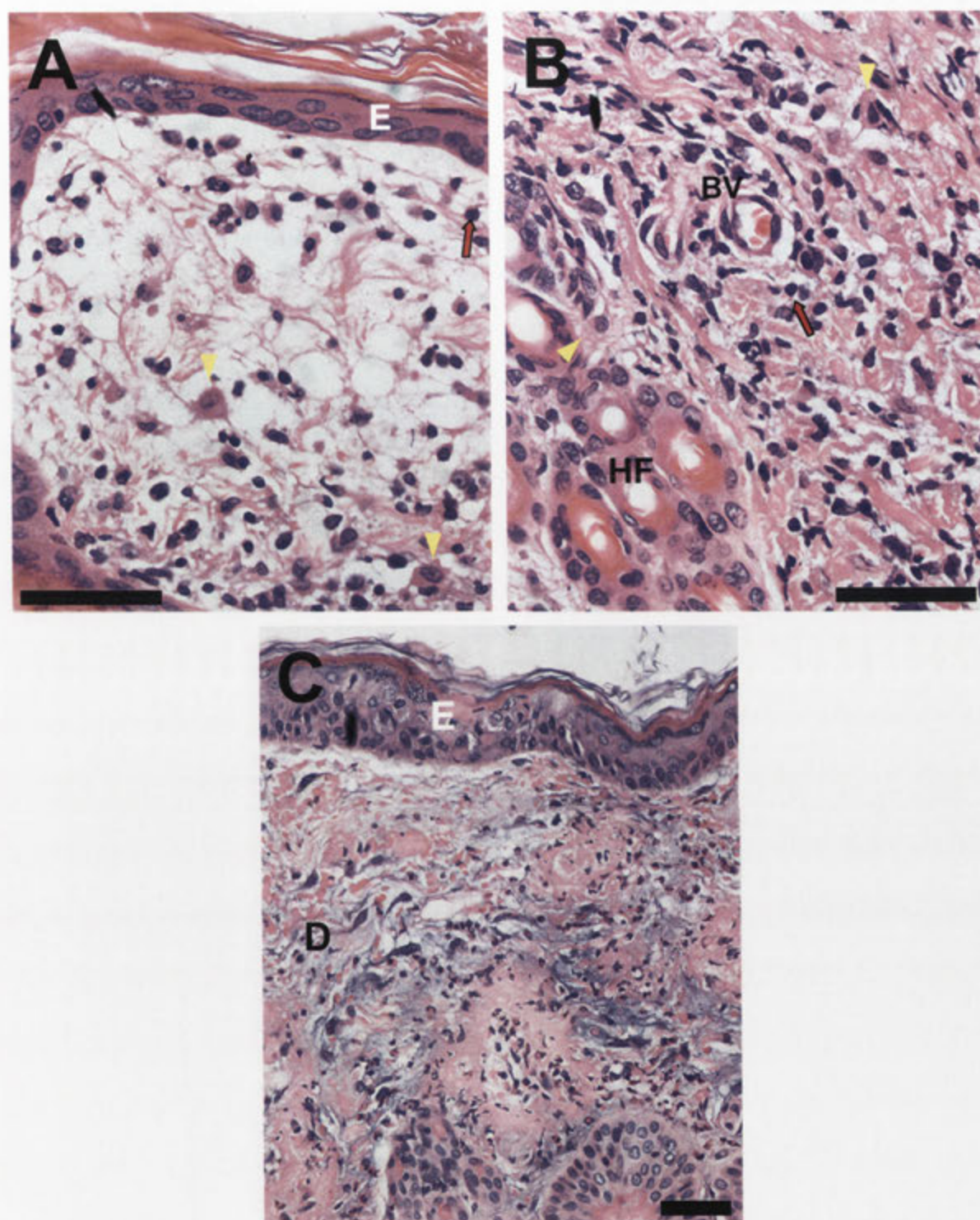
Abbreviations: PMN, polymorphonuclear cells.





**Figure 5.14 - Histopathology of wild rabbit skin at the inoculation site after TKO infection.** Skin section from a rabbit 4 days after TKO infection (A). Skin sections from rabbits 6 days after TKO infection (B-D). **A:** Skin at 4 dpi contained numerous lymphocytes near the epidermis. **B:** The epidermis and upper dermis at 6 dpi with a predominantly polymorphonuclear cell infiltrate. **C:** The epidermis and upper dermis of another rabbit at 6 dpi with a predominantly mononuclear cell infiltrate. **D:** Lymphocytes and macrophages adjacent to a blood vessel in the deeper dermis at 6 dpi. Bars on images are 50  $\mu$ m in length. Key: E, epidermis; BV, blood vessel; HF, hair follicle; black arrowhead, polymorphonuclear cell; yellow arrowhead, macrophage; red arrow, lymphocyte; NF, nuclear fragment (apoptotic body). Examples of each cell type and structure are indicated.





**Figure 5.15 - Histopathology of wild rabbit skin at the inoculation site 10 days after myxoma virus infection.** Skin sections from TKO-infected rabbits (A and B). Skin section from a Uriarra-infected rabbit (C and D). **A:** The epidermis and upper dermis of a TKO-infected rabbit. **B:** The deeper dermis of a TKO-infected rabbit. **C:** Epidermis and upper dermis of a Uriarra-infected rabbit. Bars on images are 50  $\mu$ m in length. Key: E, epidermis; D, dermis; BV, blood vessel; HF, hair follicle; yellow arrowhead, macrophage; red arrow, lymphocyte. Examples of each cell type and structure are indicated.

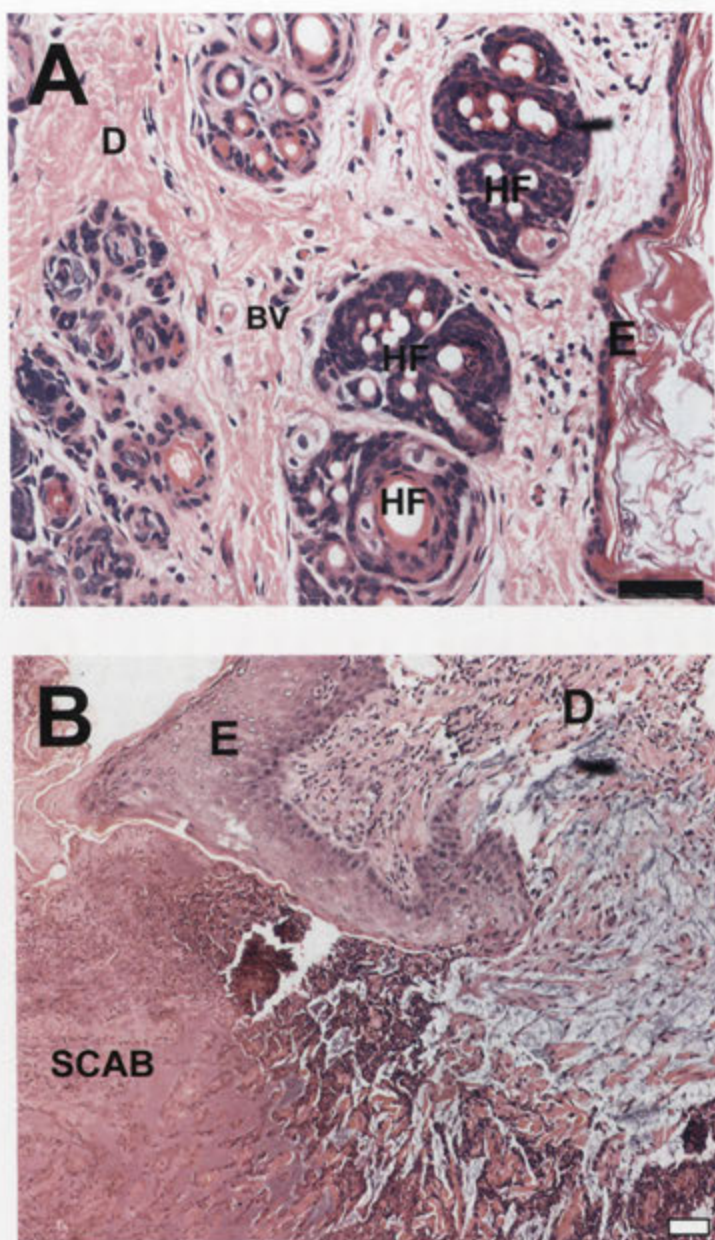


with much of the tissue filled with pink-staining material and fibrin fibres (Figure 5.15C). By 20 dpi, the skin of TKO-infected wild rabbits had returned to normal appearance and thickness (0.5-1.5 mm; Figure 5.16A). As seen in domestic rabbits, the skin of Uriarra-infected wild rabbits was still very abnormal and degraded, and covered by a very thick scab (Figure 5.16B).

#### *5.2.2.3 Histopathological changes in the draining lymph node of wild rabbits following TKO infection*

Haematoxylin and eosin-stained sections of the draining lymph node from three TKO-infected wild rabbits at each time point were examined. Sections from Uriarra-infected rabbits prepared in a previous study (Best *et al.*, 2000; Best and Kerr, 2000) were re-examined and compared. The histological changes seen in the draining lymph node of TKO- and Uriarra-infected rabbits are summarised in Table 5.4.

The histopathological changes seen in the draining lymph nodes of wild rabbits infected with TKO or Uriarra showed a similar progression to domestic animals infected with each virus (Section 5.2.1.2.4). There were two major differences in the draining lymph node of wild rabbits compared to domestic rabbits: 1 - a reduced presence of polymorphonuclear cells and eosinophilic mononuclear cells at 2 dpi during both TKO and Uriarra infections and; 2 - very little depletion of lymphocytes in the draining lymph nodes of TKO-infected rabbits at any time after infection. Uriarra-infected rabbits showed some reduction in lymphocyte density at 6 and 10 dpi, but it was not as extensive as that seen in Uriarra-infected domestic rabbits, and there was no evidence of the apoptosis seen in domestic rabbits at 6 dpi.



**Figure 5.16** - Histopathology of wild rabbit skin at the inoculation site 20 days after myxoma virus infection. **A:** Skin section from a TKO-infected rabbit. **B:** Skin section from a Uriarra-infected rabbit that was covered by a thick scab. Bars on images are 50 µm in length. Key: E, epidermis; D, dermis; HF, hair follicle; BV, blood vessel. Examples of each cell type and structure are indicated.

**Table 5.4 - A summary of the histopathological changes of wild rabbit draining lymph nodes following infection with TKO or Uriarra.**

TKO		Uriarra
Two Days Post Infection		
Cortex	Several small secondary follicles with distinct germinal centres	Several secondary follicles with no distinct germinal centres
Cellular infiltrate	Normal	Normal
Paracortex	Normal	Normal
Four Days Post Infection		
Cortex	Increased numbers of secondary follicles since 2 dpi; germinal centres darkly stained; haemorrhaging in the cortex	Large increase in number of active germinal centres, found into paracortex
Cellular infiltrate	Macrophages in cortex and subcapsular sinus	Macrophages in cortex and subcapsular sinus
Paracortex	Some haemorrhaging, otherwise normal	Normal
Six Days Post Infection		
Cortex	Large secondary follicles with dark germinal centres	Large secondary follicles with dark germinal centres
Cellular infiltrate	No change	Increased numbers of macrophages in paracortex and germinal centres
Paracortex	Very slight depletion of lymphocytes compared to uninfected lymph nodes; mitotic cells and macrophages present	Slightly lower density of lymphocytes compared to uninfected lymph nodes; macrophages present
Ten Days Post Infection		
Cortex	Numerous large germinal centres throughout cortex and paracortex; depletion of lymphocytes in germinal centres; apoptotic cells; macrophages with apoptotic bodies prevalent	Large germinal centres throughout cortex and paracortex; depletion of lymphocytes in germinal centres; macrophages with apoptotic bodies present
Cellular infiltrate	Macrophages in sinuses	No change from 6 dpi
Paracortex	Normal; macrophages present	Slightly lower density of lymphocytes compared to uninfected lymph nodes; more numerous macrophages
Twenty Days Post Infection		
Cortex	Numerous large active germinal centres in cortex; macrophages present	Very large germinal centres; cortex depleted of lymphocytes; macrophages surrounded secondary lymphoid follicles and within germinal centres
Cellular infiltrate	Normal	Large numbers of macrophages in all parts of the lymph node
Paracortex	Normal	Normal

Abbreviations: PMN, polymorphonuclear cells.

### 5.3 DISCUSSION

The pathogenesis of TKO in domestic rabbits was studied to further evaluate the suitability of the virus as a vaccine to protect domestic rabbits from myxomatosis. This study allowed us to evaluate the level of tissue damage occurring in the skin and draining lymph nodes of TKO-infected rabbits, the potential transmissibility of the vaccine virus, its *in vivo* dissemination and the effects of TKO infection on the fertility of vaccinated male rabbits. The results of this pathogenesis study will be discussed with respect to the suitability of TKO as a vaccine for use in protecting domestic rabbits in Australia from myxomatosis.

#### 5.3.1 The pathology of TKO infection in domestic rabbits

The histopathological changes seen in skin at the inoculation site of TKO-infected rabbits were very similar to those seen following infection with the more virulent Uriarra strain of myxoma virus. However, two important differences in the infection were seen. Although the degradation of the dermis seen within the primary lesion during the first 10 days of infection was similar with both viruses, the sizes of the lesions were very different. TKO lesions were no larger than 20 mm in diameter, while Uriarra lesions were up to 60 mm in size - 9 times the area. Secondly, TKO-induced lesions were resolved much more rapidly than Uriarra-induced lesions. By 20 dpi, the skin at the inoculation site of TKO-infected rabbits was of normal structure and appearance, while Uriarra-infected skin was still swollen, highly degraded and contained an extensive inflammatory cell infiltrate.

There was very little pathology observed in the draining lymph node of TKO-infected domestic rabbits. The histopathological changes that were observed (swelling of the lymph node, formation of germinal centres) were indicative of a robust immune response, rather

than the virus-induced damage seen during infection with more virulent strains of myxoma virus (Best *et al.*, 2000).

The rapid control and clearance of TKO compared to Uriarra coincided with a change in the nature of the inflammatory cell infiltrate in the skin. Between 6 and 10 dpi, the infiltrate in TKO-infected skin changed from predominantly polymorphonuclear cells, to an extensive mononuclear cell infiltrate. In this time, TKO titres in the skin dropped by a factor of 10,000. In contrast, the infiltrate in Uriarra-infected skin was unchanged, consisting primarily of polymorphonuclear cells. Uriarra titres were likewise unchanged. The exact composition of the cellular infiltrate in TKO-infected skin cannot be confirmed without immunohistochemical analysis of tissue sections to identify specific cellular markers. However, the morphology of cells observed in the skin at 10 dpi indicate that large number of lymphocytes were present, which most likely included virus-specific CTLs, as these are detectable by 4 days after infection of mice with ectromelia virus (Blanden and Gardner, 1976) or sheep with VV (Issekutz, 1984).

The deletion of M010L, M011L and M-T7 in the genome of the TKO vaccine virus was probably responsible for the changes in the magnitude and character of the cellular infiltrate in TKO-infected skin, the rapid clearance of the vaccine virus and the limited pathology in the draining lymph node, compared to Uriarra infection. Previous studies have shown that the deletion of M011L increased the magnitude of the inflammatory infiltrate in infected skin compared to wild-type virus infection (Opgenorth *et al.*, 1992). This was due to the apoptosis of infected macrophages, normally prevented by M011L expression (Everett *et al.*, 2000), resulting in higher levels of the pro-inflammatory cytokine IL-1 $\beta$  in infected skin (Savill, 1997). The inability of TKO to replicate in lymphocytes, the most common

cell type in the lymph node, limited the amplification of TKO in the lymph node leading to reduced pathology in the draining lymph node, and the very low dissemination of the virus to other tissues. Deletion of M-T7 has been shown to increase numbers of mononuclear cells including CD43<sup>+</sup> T cells aggregating at the epidermal/dermal border where virus replication occurred (Mossman *et al.*, 1996b), as the chemokine gradients required for efficient migration of lymphocytes were present (Laing and Secombes, 2004). A more robust cellular immune response was also evident in secondary lymphoid organs such as the lymph nodes, due to higher levels of bioactive IFN- $\gamma$  (Mossman *et al.*, 1996b). Both of these effects were seen in TKO-infected skin and lymph nodes.

Although there was marked pathology in the skin of TKO-infected rabbits, normal pathology was restored by 20 dpi, suggesting that TKO will be suitable as a vaccine for domestic rabbits. Once virus had been cleared from the tissue, there was no longer any viral antigen stimulation required for the maintenance of an inflammatory response and the activity of CTL populations (Esser *et al.*, 2003). The inflammatory response in the skin of TKO-infected domestic rabbits was rapidly curtailed between 12 and 15 dpi, visible by the reduced inflammation and redness of skin at the edges of the primary lesion and a return to normal skin colour (Chapter 4). This allowed a reduction in swelling, the swift repair of skin tissue in the lesion and the establishment of normal pathology by 20 dpi.

The long-term effects to the skin at the inoculation site following TKO vaccination have not been evaluated as the fur was removed from the inoculation site before infection. Secondary lesions induced by Uriarra  $\Delta$ M10L/M11Lgpt and Uriarra  $\Delta$ M-T7gpt caused long-term scarring and alterations in fur regrowth (Chapter 4) and similarly unacceptable effects may occur at TKO-induced primary lesions. This will require further investigation

through the vaccination of rabbits without clipping of the fur at the inoculation site, as would occur in a clinical environment.

This study did not detect TKO by plaque assay in the testes of domestic rabbits following infection, and histological analysis of testes revealed no abnormalities at any time up to 20 dpi. Therefore, it can be concluded that vaccination with 5000 pfu of TKO does not result in the dissemination of the vaccine virus to the testis, and would be highly unlikely to have any effect on the fertility of male domestic rabbits.

### **5.3.2 Potential transmissibility of TKO in domestic and wild rabbits**

There is the formal possibility that TKO could be transmitted from recently vaccinated domestic rabbits to wild rabbits by arthropod vectors because two domestic rabbits had titres at the inoculation site greater than  $10^7$  pfu/g at 6 dpi, the level considered transmissible by mosquitoes (Fenner *et al.*, 1956). However, at both 4 and 10 days, the titres were below this limit, suggesting that TKO was only at transmissible levels for 3-5 days, and possibly as little as 2 days. Previously described studies have shown that Uriarra had titres greater than  $10^7$  pfu/g in half of infected rabbits at 4 dpi, and in all rabbits at 6 and 10 dpi, having achieved maximum titres 100-fold greater than TKO (Best and Kerr, 2000), and was readily transmissible by mosquitoes between 6 and 18 dpi (Fenner *et al.*, 1952; Fenner and Ratcliffe, 1965).

TKO titres in wild rabbits were investigated to determine if the virus was capable of reaching transmissible titres in genetically resistant animals and could be transmitted and maintained amongst wild rabbit populations. This possibility is the primary reason myxoma virus vaccines are not permitted in Australia (Fenner and Ross, 1994). Although

this formal possibility exists, it is highly unlikely for three reasons. Firstly, the virus reached transmissible titres in only 1/3 of rabbits at 4 dpi, and was below transmissible levels at both 2 and 6 dpi. Secondly, genetically resistant rabbits had an earlier, more intense mononuclear cell infiltrate into the inoculation site compared to domestic rabbits, which may have been responsible for the more rapid control of TKO in the skin as seen by the lower peak titres and a drop in virus titres at 6 dpi. Thirdly, mildly attenuated viruses, which are virulent enough to reach high titres in the skin and disseminate to secondary skin sites, but are attenuated enough to allow for extended survival periods, are more successful in the field in Australia. This is because virus is transmissible by arthropod vectors for longer than virulent strains (due to the death of the host) and highly attenuated strains (due to the clearance of the virus). This has resulted in mildly attenuated strains of myxoma virus (grade III and IV) becoming predominant in Australia (Fenner and Marshall, 1957; Kerr and Best, 1998). Recent studies suggest that selective advantage is shifting toward even more virulent (grade I and II) virus strains (Kerr *et al.*, 2003; Saint *et al.*, 2001). As TKO is only potentially transmissible in genetically resistant animals for at most 2-3 days, and in a small proportion of infected animals, it is highly unlikely to successfully compete with the more virulent myxoma virus strains in the field and become established in wild rabbit populations.

### 5.3.3 Conclusions

Following infection with TKO, domestic rabbits showed only mild and transient pathology at the inoculation site, and very little virus-induced pathology at the draining lymph node, suggesting that this vaccine will be acceptable for use in domestic rabbits. However, of concern was the potential transmissibility of the TKO virus from vaccinated domestic rabbits, and between wild rabbits, indicated by the ability of the virus to reach titres of



greater than  $10^7$  pfu/g in the skin at the inoculation site. Although the establishment of TKO in wild rabbit populations is unlikely, it remains a formal possibility, rendering TKO potentially unacceptable as a vaccine for use in Australia according to the criteria established in Section 1.6.

In the following chapter, the efficacy and acceptability of TKO in younger animals will be described, a study that was conducted concurrently with the TKO pathogenesis study described in this chapter.

## **Chapter 6**

# **Evaluation of TKO's safety and efficacy in younger domestic rabbits**

## **CHAPTER 6 – EVALUATION OF TKO'S SAFETY AND EFFICACY IN YOUNGER DOMESTIC RABBITS**

### **6.1 INTRODUCTION**

Concurrently with the pathogenesis study described in Chapter 5, TKO was tested in younger domestic rabbits (8 and 12 weeks old) to determine its safety in these age groups. Rabbits of this age are a more likely target of a commercial myxomatosis vaccine, and younger rabbits often have a lower tolerance for attenuated myxoma virus vaccines, resulting in increased incidence of vaccine-induced disease, generalised immune suppression and the establishment of potentially fatal secondary bacterial infections (McKercher, 1952; Vautherot *et al.*, 1997). This chapter describes the investigation of the clinical response of 8 and 12 week old rabbits to TKO, their anti-myxoma virus antibody responses and the protective efficacy of the vaccine. This has allowed us to determine if TKO is suitable for use in rabbits of this age group. The vaccine was tested at two doses. Twelve week old rabbits were vaccinated with 5000 or 50 pfu of TKO, whilst eight week old animals were vaccinated with 50 pfu of the vaccine virus.

### **6.2 RESULTS**

#### **6.2.1 Inoculation of 12 week old rabbits with 5000 pfu of TKO**

##### *6.2.1.1 Vaccination of rabbits*

Six 12 week old, male domestic rabbits were inoculated with 5000 pfu of TKO on the left thigh and examined daily for 14 days following vaccination. A summary of the clinical signs of these animals is recorded in Table 6.1. This dose of TKO had been well tolerated in mature rabbits with no vaccine-induced secondary lesions observed, as described in Chapter 4.

**Table 6.1** – Clinical signs of 12 week old domestic rabbits following vaccination with 5000 pfu of TKO.

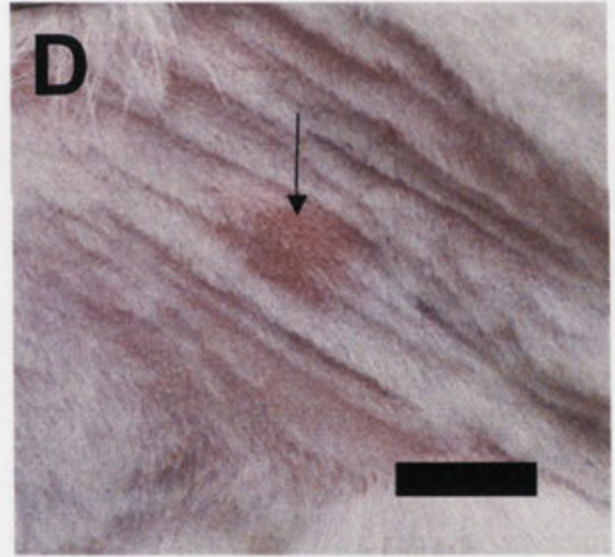
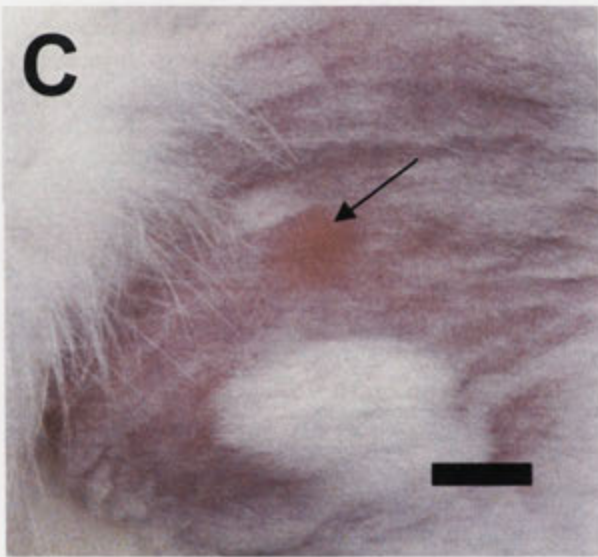
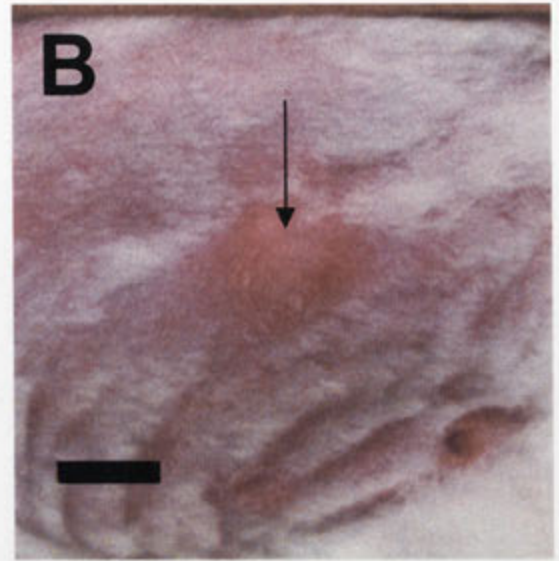
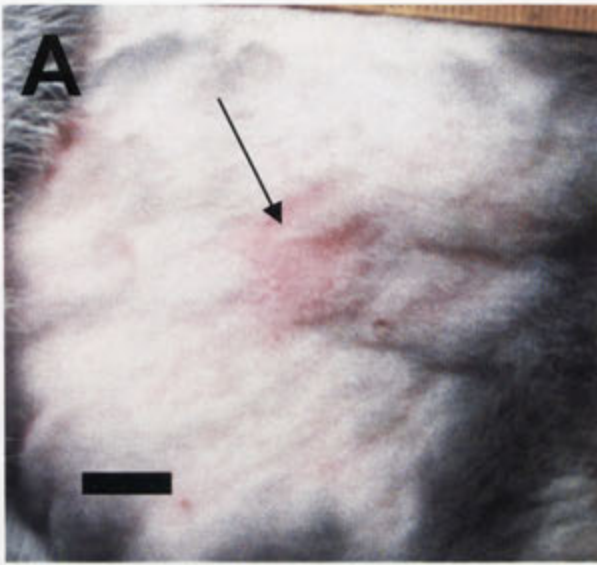
Day post inoculation	Clinical signs
2	Slight reddening of skin at the inoculation site
3	Primary lesion was a soft, pink lump 6-12 mm in diameter.
5	Primary lesion 10-20 mm in diameter, red and raised from surrounding skin.
7	Primary lesion 15-30 mm in diameter, red and protuberant. Single, small secondary lesion on the edge of the ear of two rabbits (211 and 229).
8	Primary lesion crimson or red in colour with darker haemorrhagic patches forming in centre. Secondary lesions forming on the edge of the ears of three rabbits (237, 211 and 229). Secondary lesions were red, ~5 mm hard lumps.
10	Primary lesion circumscribed, 15-30 mm in diameter, scabbed over and haemorrhagic. Secondary lesions on 4/6 rabbits (237, 211, 227 and 229), primarily on margins of ears. One animal (229) also had a lesion on the anus and one lesion on eyelid.
14	Primary lesion covered with a hard, black, concave scab, with skin at edges faded from red to pink in colour. Secondary lesions scabbed over and regressing.

Within 3 days post infection (dpi), a reaction was seen at the inoculation site on all rabbits as a 6-12 mm pink lump. By 4 dpi, the primary lesion had grown to an 8-15 mm raised pink lump on all animals (Figure 6.1). By 9 dpi, four rabbits had several small secondary lesions on the edges of the ears and one of these rabbits (rabbit 229) also had a very small (~3 mm) secondary lesion on the eyelid, and a secondary lesion on the anus. The primary lesions had become circumscribed, and had haemorrhagic patches at the centre. By 12 dpi, the primary lesions on all animals were scabbed over and regressing slightly (Figure 6.2A and B), and secondary lesions had also scabbed over and begun regressing (Figure 6.2C). By 21 dpi, rabbits had completely recovered from infection except for residual scabbing at the primary lesion. These symptoms were slightly worse than those seen in mature (6-12 month old) rabbits following vaccination with 5000 pfu of TKO (Chapter 4).

Rectal temperatures for each rabbit were recorded daily. Half of the rabbits did not show increases in temperature during the 14 days post vaccination (Figure 6.3). The other three rabbits had one day at or over 40°C, including rabbit 237 which had a temperature of 41°C at 6 dpi only.

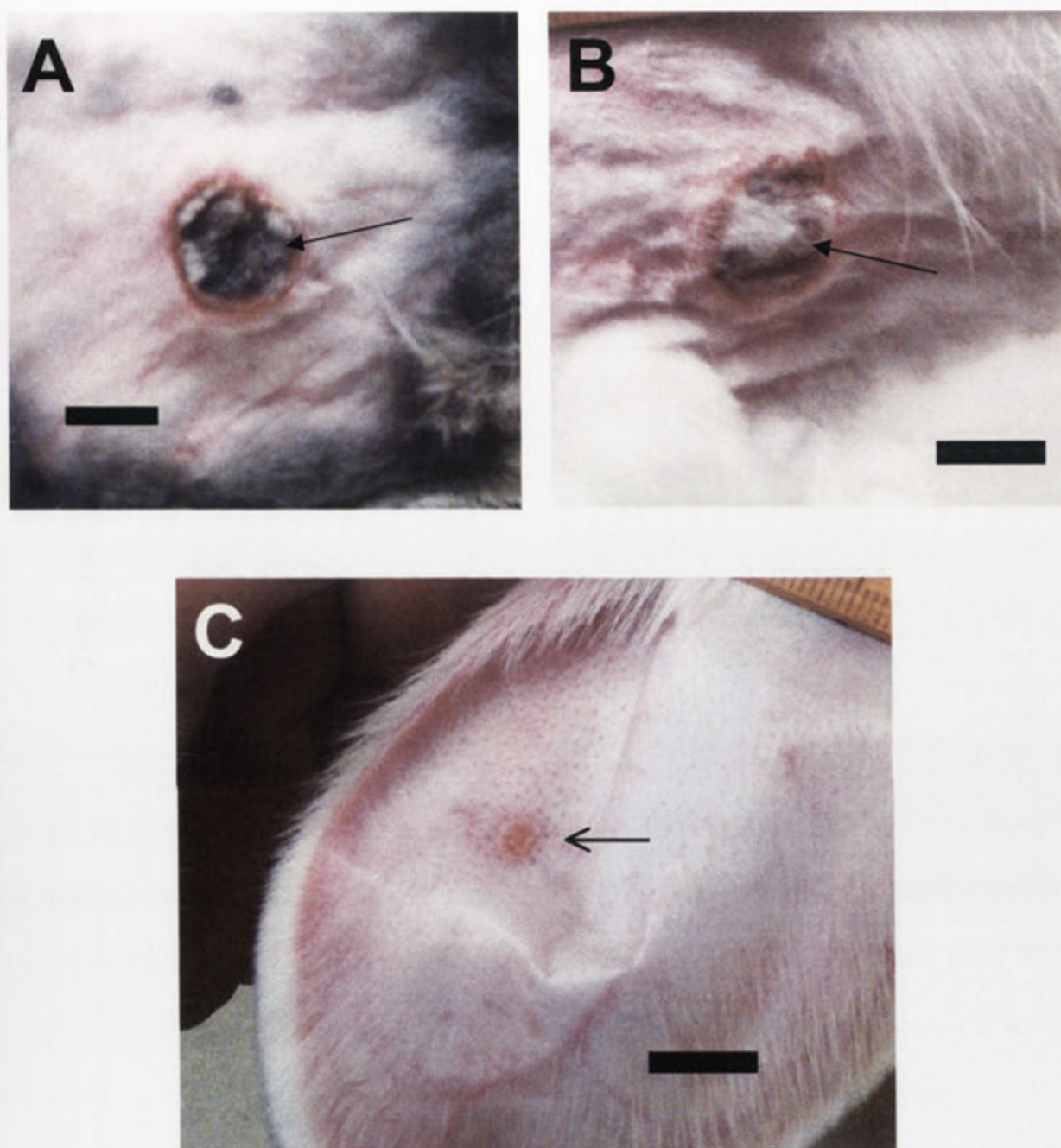
#### *6.2.1.2 SLS challenge of rabbits vaccinated with 5000 pfu of TKO at 12 weeks of age*

Rabbits were challenged with 1000 pfu of SLS on the right thigh 6 weeks after TKO vaccination and examined for 12 days following infection. A reddening at the inoculation site or the development of a small (3-5 mm) lump was seen on all rabbits at 1 day post challenge (dpc). This developed into a distinct 7-20 mm protuberant lesion by 3 dpc (Figure 6.4). The lesions on several rabbits had already developed darker, haemorrhagic regions in the centre at this point (Figure 6.4B and C). By 6 dpc, the lesion at the inoculation site had either scabbed over (Figure 6.5A-C) or had begun to fade and regress



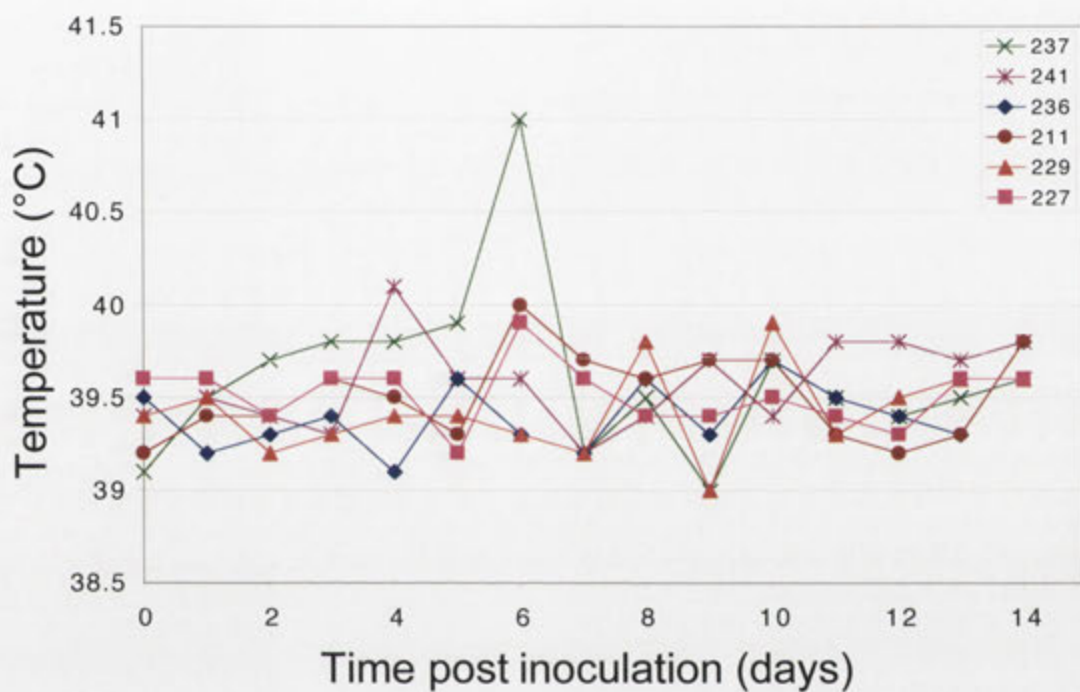
**Figure 6.1 - Inoculation sites of rabbits vaccinated with 5000 pfu of TKO at 12 weeks of age, 4 days after inoculation.** Primary lesions on rabbits were 8-12 mm in diameter, pink and slightly protuberant (A-D, arrowed). Bars on images are 10 mm in size.



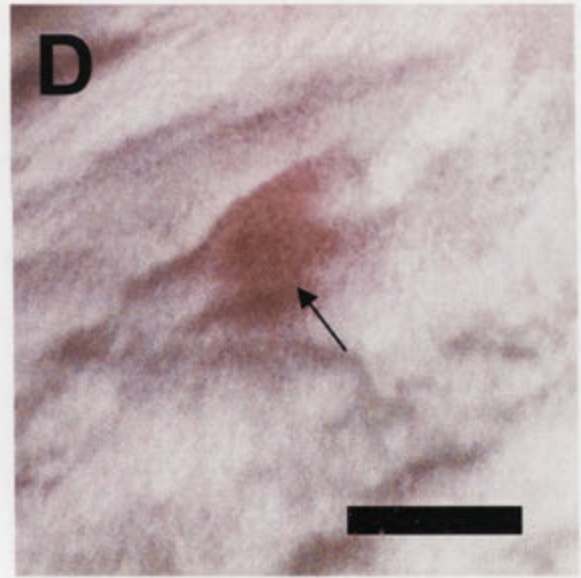
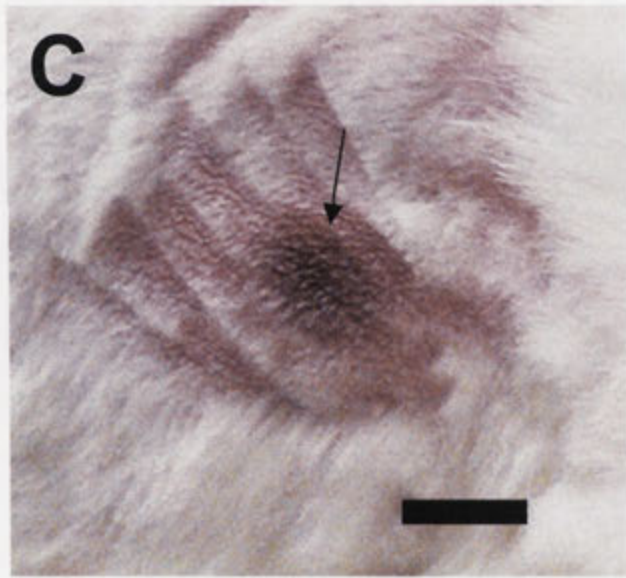
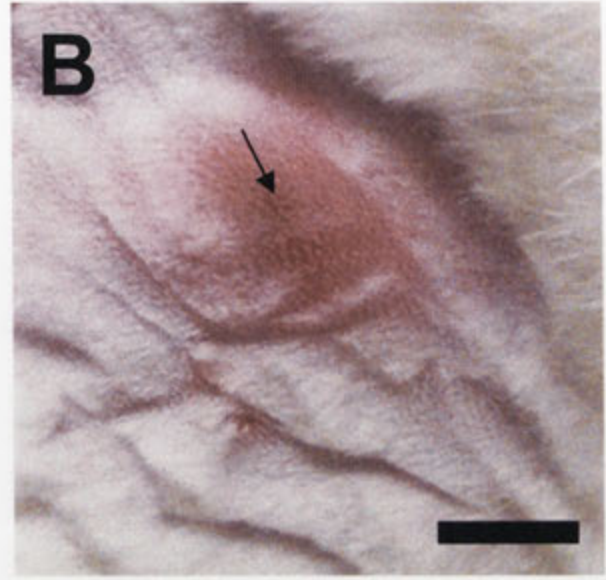
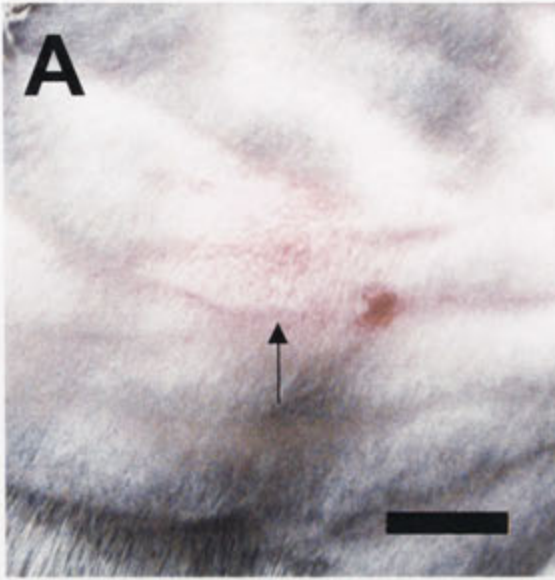


**Figure 6.2** - Primary and secondary lesions on rabbits vaccinated with 5000 pfu of TKO at 12 weeks of age, 12 days after inoculation. Representative primary lesions 10-25 mm in diameter and covered with a hard black scab are shown (A and B, arrowed). A small scabbed-over secondary lesion on the ear (C, arrowed). Bars on images are 10 mm in size.

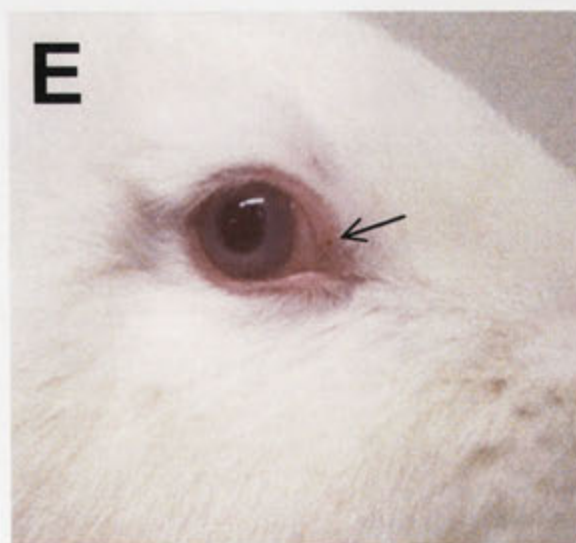
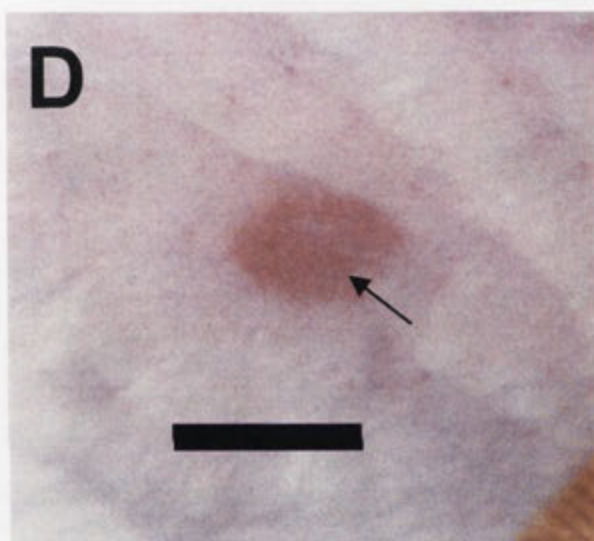
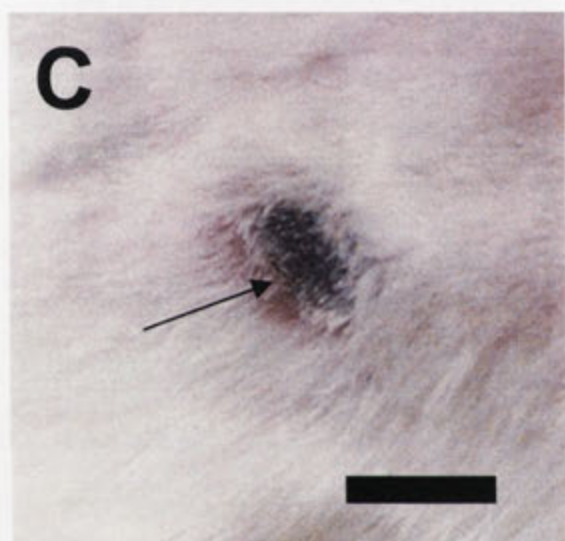
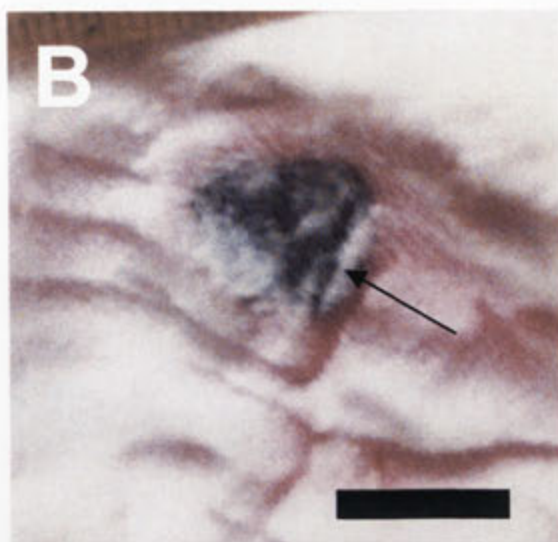
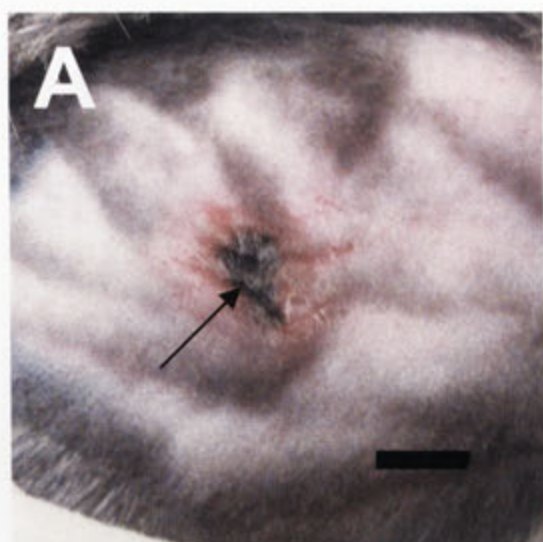




**Figure 6.3 - Rectal temperatures of 12 week old rabbits following vaccination with 5000 pfu of TKO.**



**Figure 6.4 -** Challenge inoculation site on rabbits vaccinated with 5000 pfu of TKO at 12 weeks of age, 3 days following challenge with SLS. Lesions at the site of SLS challenge are shown (A-D, arrowed) and haemorrhagic patches had developed in the centre of lesions (B and C, arrowed). Bars on images are 10 mm in size.



**Figure 6.5** - Appearance of primary and secondary lesions on rabbits vaccinated with 5000 pfu of TKO at 12 weeks of age, 6 days following challenge with SLS. Lesions at the inoculation site had scabbed over (A-C, arrowed), or begun to regress (D, arrowed). Small secondary lesion on the eyelid that had turned black and scabbed over (E, arrowed). Bars on images are 10 mm in size

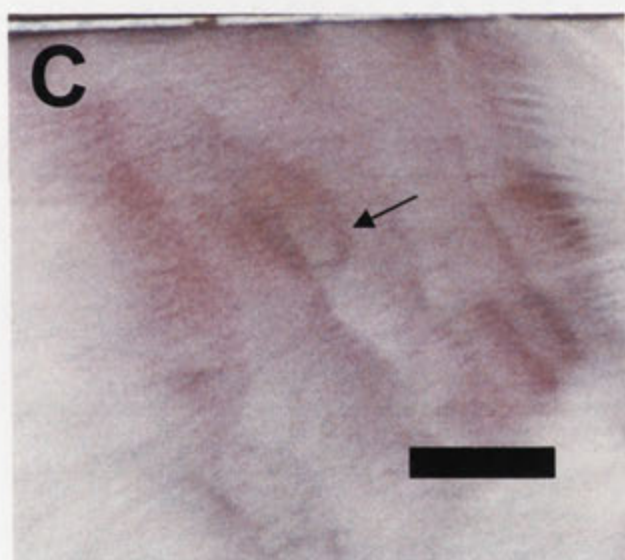
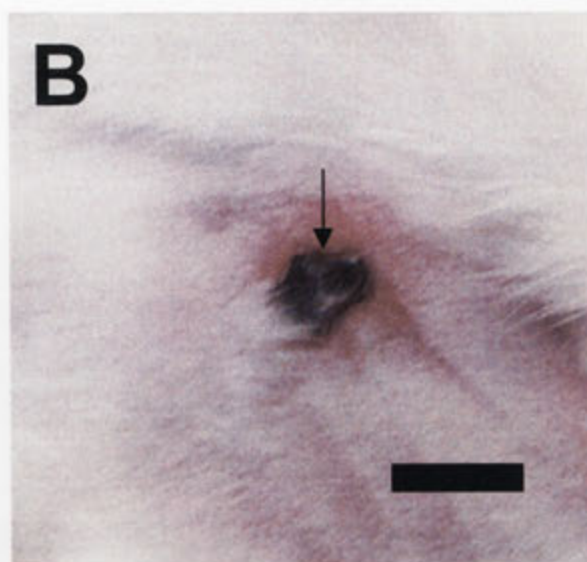
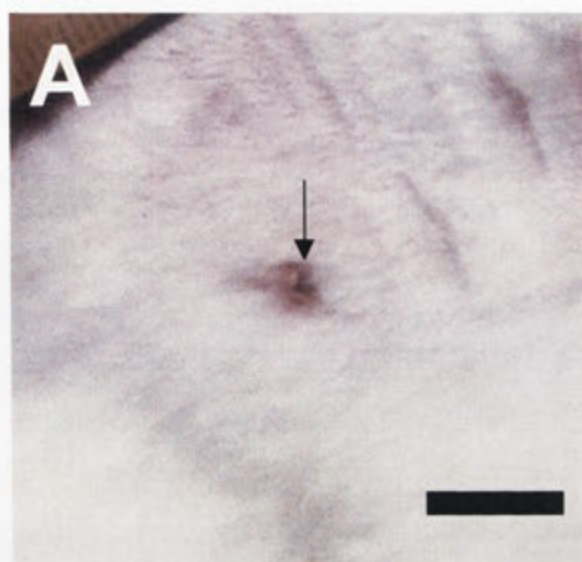
(Figure 6.5D). A single small (2-3 mm) secondary lesion was seen on the right eyelid of one rabbit (227) at 5 dpc, which had changed colour to black by 6 dpc (Figure 6.5E). By 14 dpc, this secondary lesion had completely regressed (Figure 6.6D). Lesions at the inoculation site had faded with residual scab material remaining on normal coloured skin (Figure 6.6A and B), or had regressed completely (Figure 6.6C). Three rabbits had a rectal temperature above 40°C for a single day only (Figure 6.7). All rabbits survived challenge.

#### 6.2.1.3 Analysis of anti-myxoma virus antibody responses

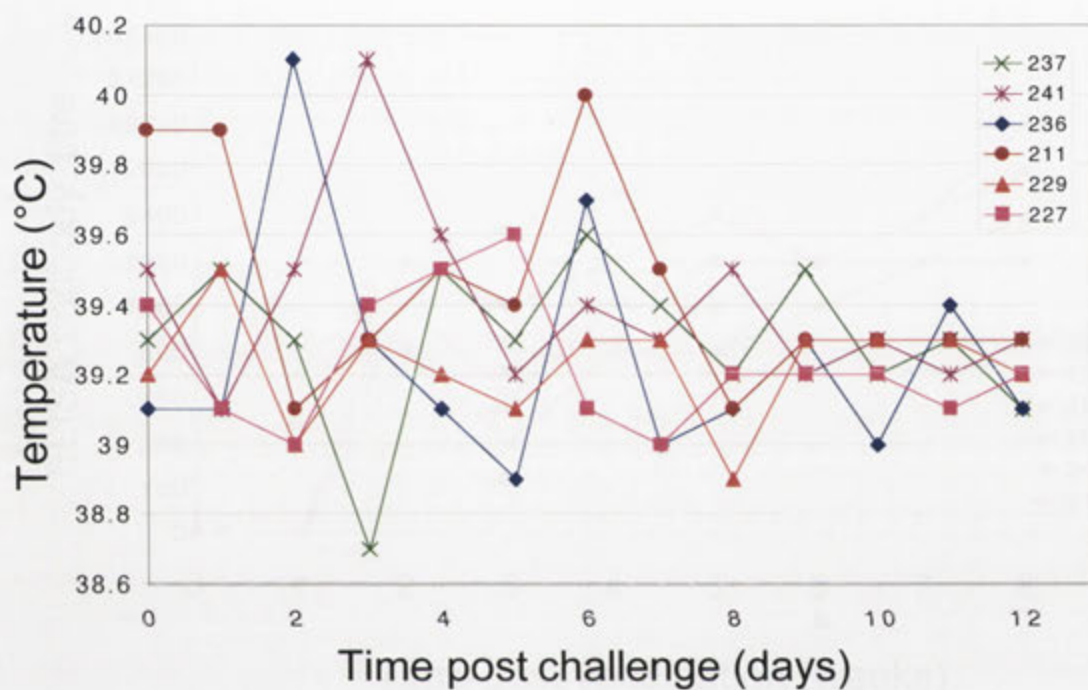
The anti-myxoma virus ELISA antibody titre was determined for all serum samples after vaccination and following challenge with SLS (Figure 6.8). The level of myxoma virus neutralising antibody was determined at 6 weeks after vaccination (day of challenge) and 8 weeks after vaccination (2 weeks post challenge) using a plaque reduction neutralisation assay (PRNA). The percentage neutralisation at each serum dilution and the 50% and 80% neutralisation titres are shown in Figure 6.9. Anti-myxoma virus antibody was first detected by ELISA in rabbits 2 weeks post vaccination (wpv), with titres ranging between 800 and 3200 (Figure 6.8). By the time of challenge at 6 wpv, titres had not changed significantly, ranging between 1600 and 3200. At this point, 50% PRNA titres were between 320 and 640 (Figure 6.9).

By 2 weeks after challenge, four of the six rabbits showed a significant increase (4-fold or greater) in anti-myxoma virus ELISA antibody titres (Figure 6.8). Two of these rabbits showed an 8-fold or greater increase in ELISA titre including rabbit 227, which had developed a secondary lesion (Figure 6.6D). Five of the six rabbits displayed a  $\geq 4$ -fold 50% PRNA titre increase after challenge, with final titres ranging between 1280 and 5210

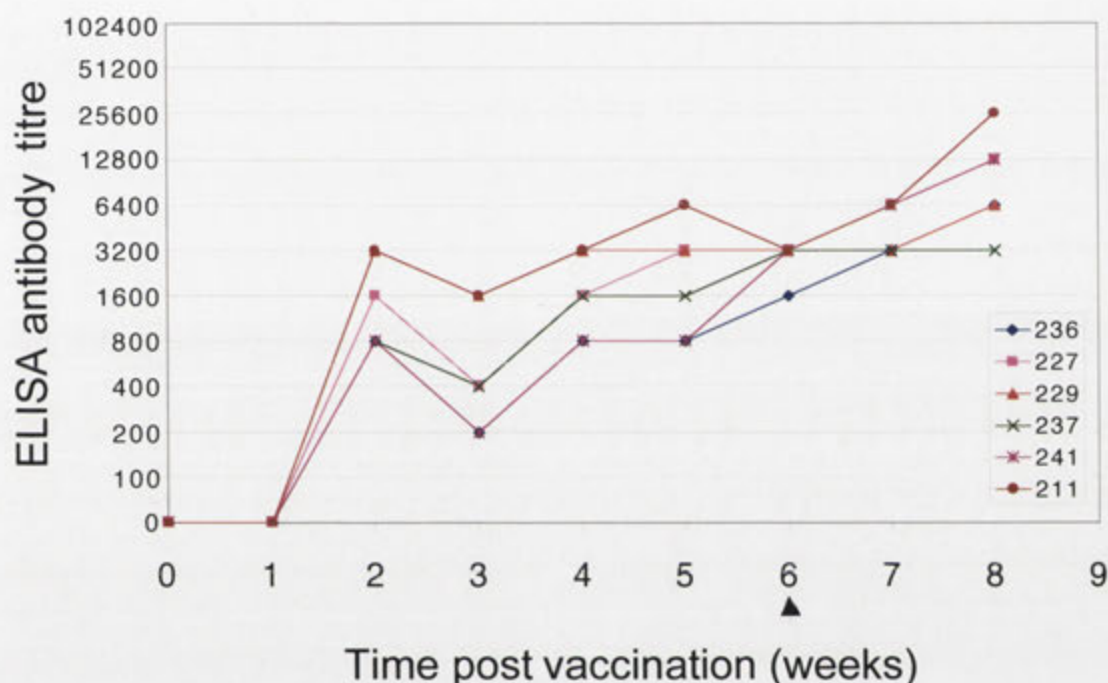




**Figure 6.6 - Appearance of primary and secondary lesions on rabbits vaccinated with 5000 pfu of TKO at 12 weeks of age, 14 days following challenge with SLS.** Lesions at the inoculation site had regressed with just a scab remaining (A and B, arrowed) or had regressed completely (C, arrowed). Secondary lesion on the eyelid of one rabbit had regressed completely (D, arrowed). Bars on images are 10 mm in size.

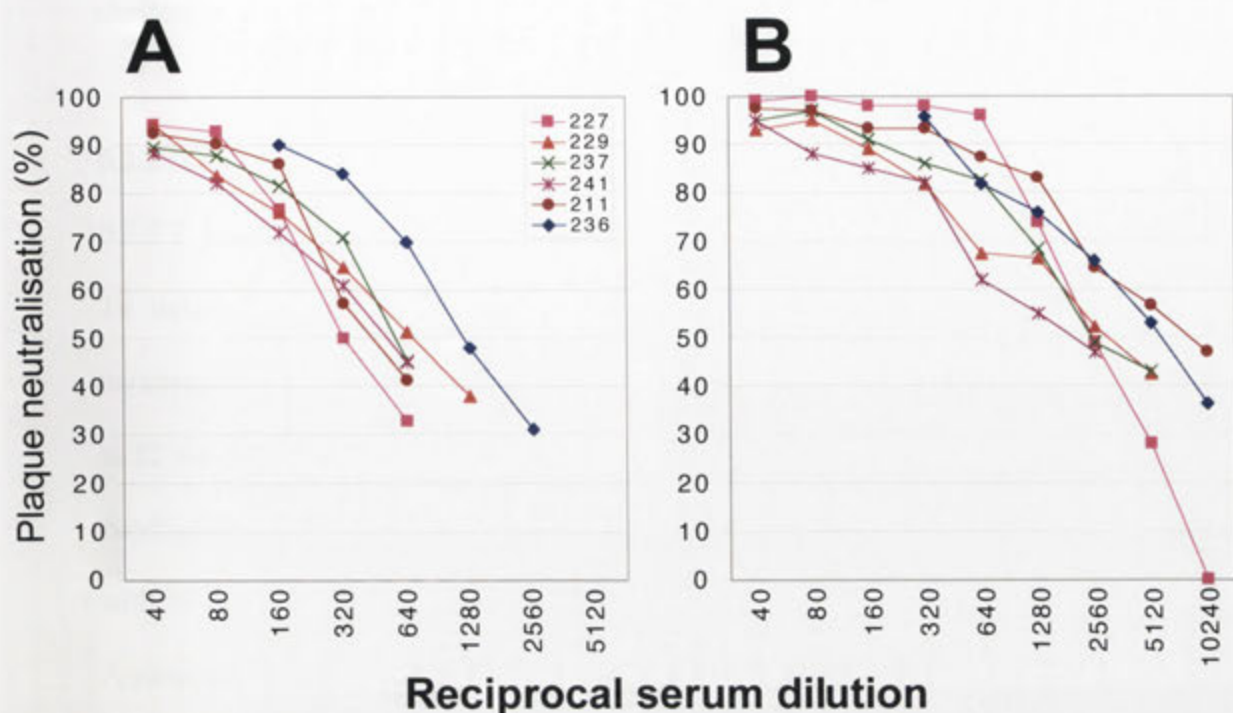


**Figure 6.7 - Rectal temperatures of 12 week old rabbits vaccinated with 5000 pfu of TKO following challenge with SLS.**



**Figure 6.8 - ELISA anti-myxoma virus antibody titres in rabbits vaccinated with 5000 pfu of TKO at 12 weeks of age.** The ELISA titre was determined by titration in duplicate of two-fold serial dilutions of rabbit sera from 1:100. The ELISA endpoint was defined as the reciprocal serum dilution 0.1  $OD_{405}$  units above the  $OD_{405}$  of the 1:100 negative control. A titre of zero was recorded when the  $OD_{405}$  of 1:100 diluted serum was less than 0.1. Rabbits were challenged with SLS following sera collection at week 6 (arrow).





**Figure 6.9 - Analysis of neutralising anti-myxoma virus antibodies in the serum of rabbits vaccinated with 5000 pfu of TKO at 12 weeks of age.** Neutralising antibody was measured by plaque reduction neutralisation assay and the percentage plaque neutralisation recorded at week 6 (A) and week 8 (B) for each serum dilution (two-fold from 1:40 to 1:10240). The titres at which 50% or greater or 80% or greater of myxoma virus plaque formation was neutralised is summarised (C).

(Figure 6.9). Increases in ELISA titre did not correlate with increases in PRNA titre after challenge.

## **6.2.2 Testing of a lower dose of TKO in 12 week old rabbits**

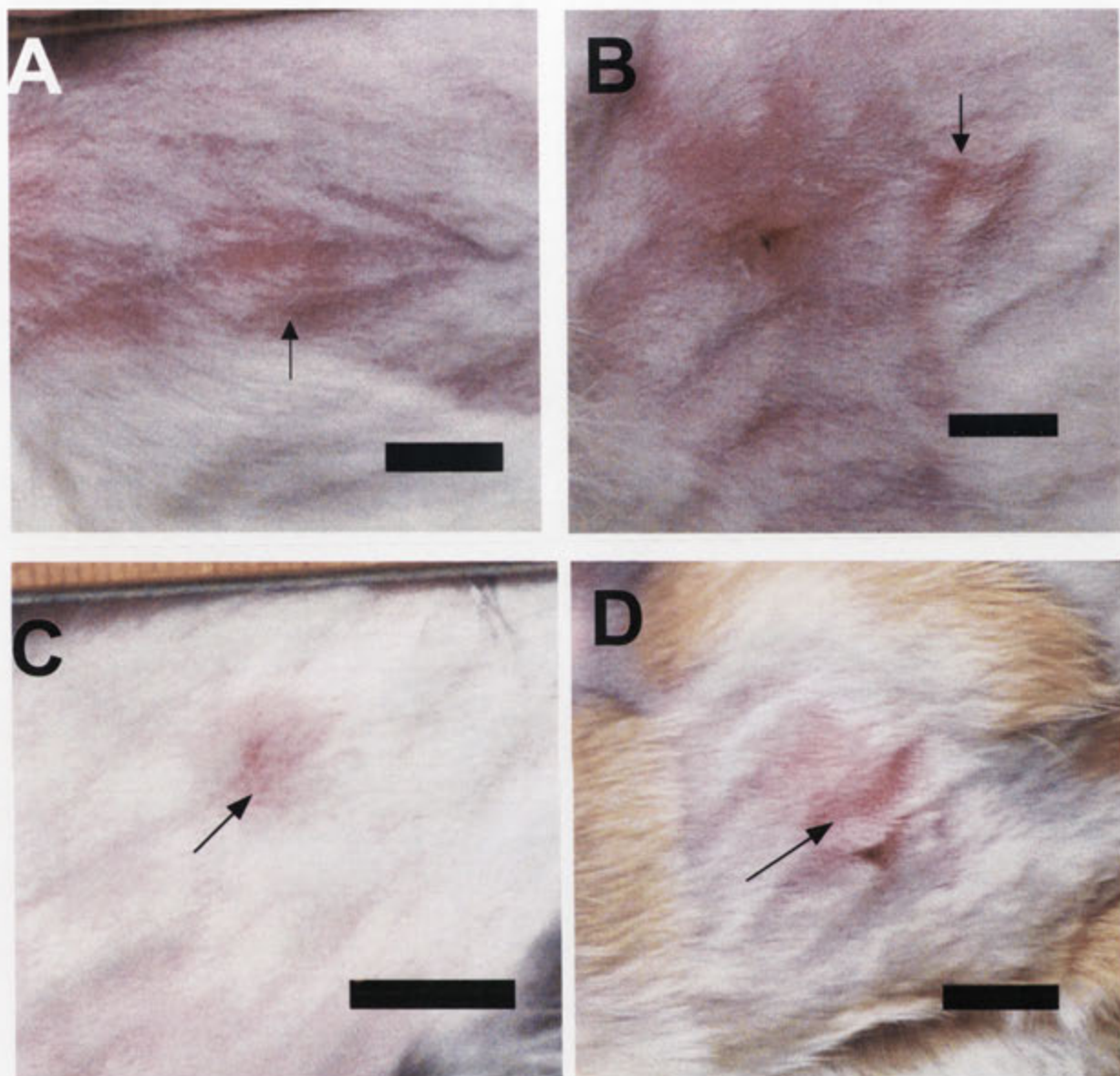
### *6.2.2.1 Inoculation of 12 week old rabbits with 50 pfu of TKO*

To determine if there was a dose-related amelioration of secondary symptoms of myxomatosis induced by the vaccine, a 100-fold lower dose of the virus was administered to 12 week old rabbits. This allowed a direct comparison between the previous experiment conducted with 5000 pfu of TKO. Six 12 week old male domestic rabbits were inoculated with 50 pfu of TKO on the left thigh, and examined for clinical signs of disease for 14 days. A summary of the clinical signs of these animals is recorded in Table 6.2.

Following inoculation, there was no response at the inoculation site until 4 or 5 dpi, 2-3 days later than found following inoculation with 5000 pfu of TKO. Lesions developed into a raised pink lump ranging in size from 7-15 mm by 6 dpi (Figure 6.10). By 8 dpi, small secondary lesions (2-4 mm) were noted on the ears or nose of three rabbits (a total of four secondaries on all three rabbits), one less than following the larger dose of virus. As found after inoculation with 5000 pfu of TKO, the primary lesions on most rabbits were circumscribed by 10 dpi, and several exhibited haemorrhagic patches or a cream coloured scabby material on the surface. Lesions ranged in size from 12 to 25 mm in diameter. Rabbits recovered quickly from this point, and by 13 dpi, the primary lesions were scabbed over (Figure 6.11A-D) or were fading and regressing. Secondary lesions were hard, scabbed over (Figure 6.11E), and no larger than 4 mm in diameter.

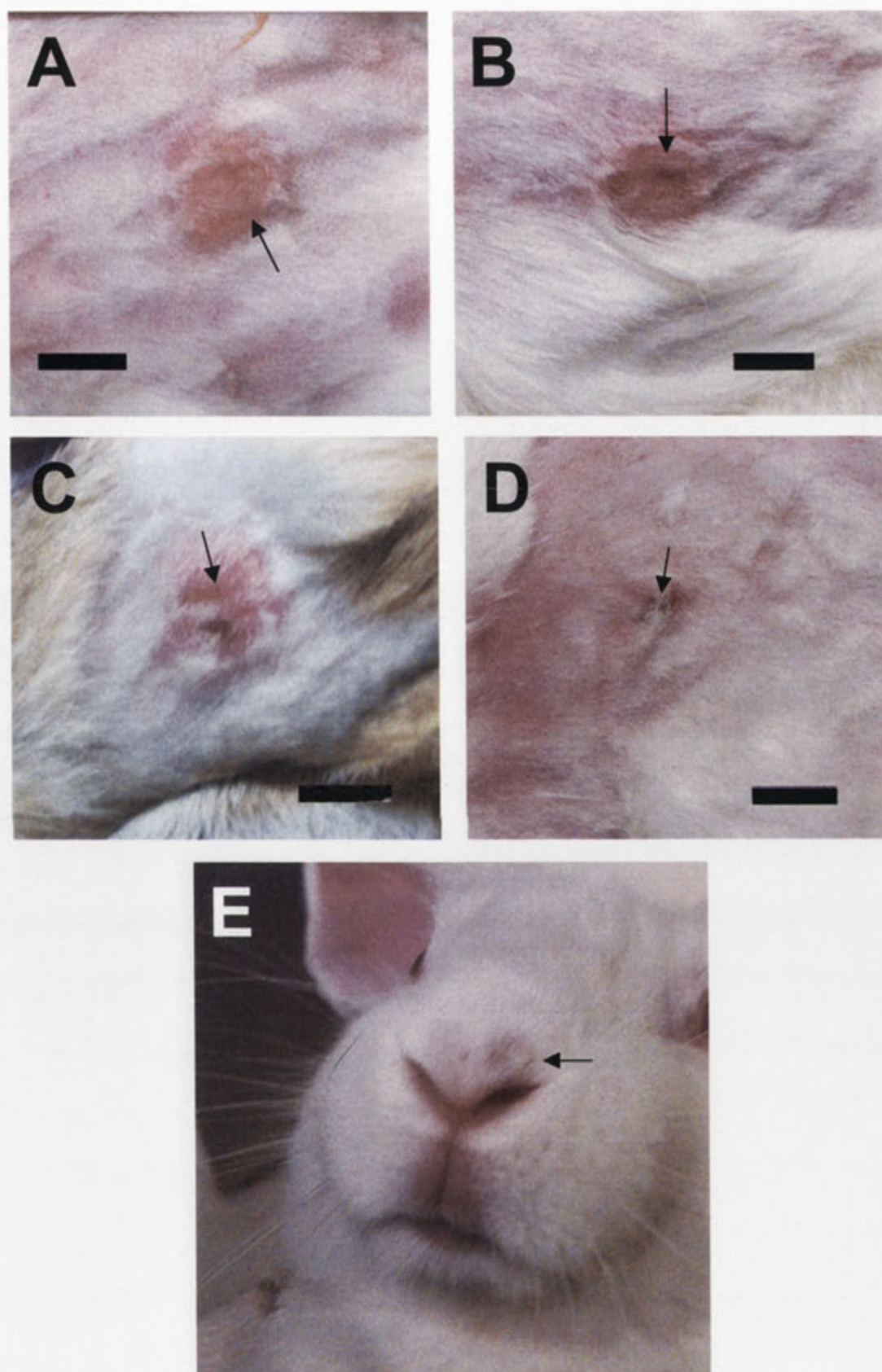
**Table 6.2** – Clinical signs of 12 week old domestic rabbits following vaccination with 50 pfu of TKO.

Day post inoculation	Clinical signs
4	Slight reddening of skin at the inoculation site
5	Primary lesions had developed on 5/6 rabbits, 4-12 mm in diameter, soft and pink in colour. One rabbit (252) had slight reddening of skin at the inoculation site.
7	Primary lesions were 14-20 mm in diameter, firm and red, except for rabbit 252 with a 5 mm pink lump.
8	Primary lesions 7-22 mm in diameter, raised, red. Secondary lesions on three rabbits (260, 247 and 242) on margins of ears, or on nose.
10	Primary lesions circumscribed, 12-25 mm diameter, crimson in colour, some with a black, haemorrhagic centre. Secondary lesions red, most beginning to scab over.
14	Primary lesion scabbed over on 4/6 rabbits and all primary and secondary lesions had begun to regress.



**Figure 6.10** - Inoculation site of rabbits vaccinated with 50 pfu of TKO at 12 weeks of age, 6 days after inoculation. Primary lesions were small and pink (A and B, arrowed); or larger and protuberant (C and D, arrowed). Bars on images are 10 mm in size.





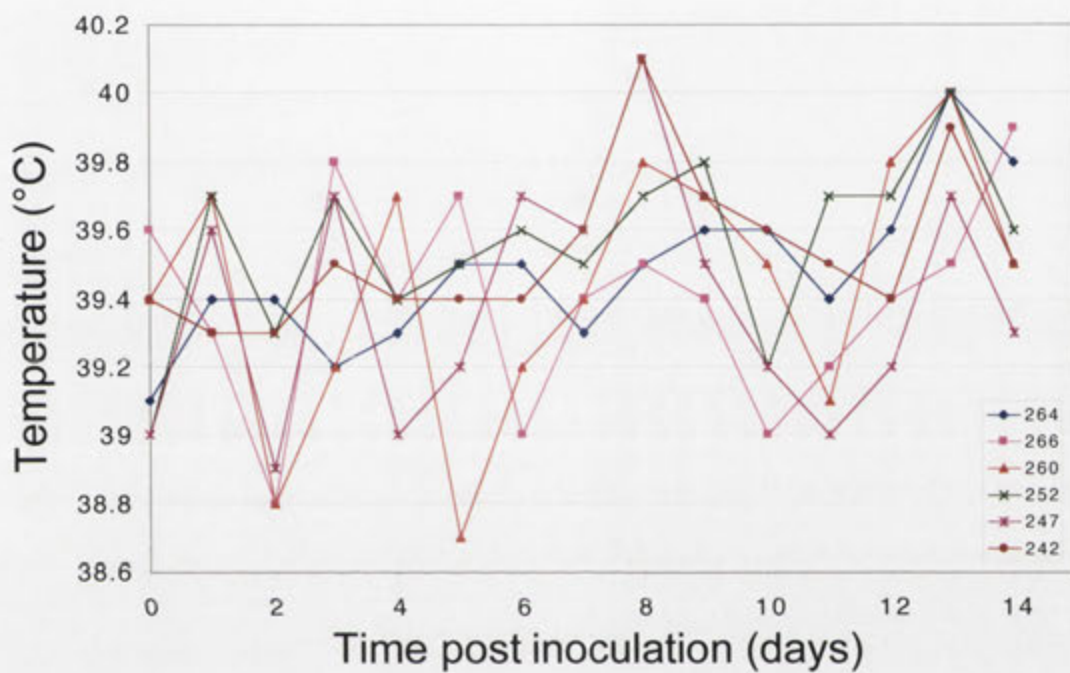
**Figure 6.11** - Primary and secondary lesions on rabbits vaccinated with 50 pfu of TKO at 12 weeks of age, 13 days after inoculation. Primary lesions with a slightly scabby surface (A-C, arrowed). Rabbit 266 had a very small primary lesion, covered with a black scab (D, arrowed). Secondary lesion on the nose of a rabbit (E). Bars on images are 10 mm in size.

The rectal temperatures of rabbits following inoculation are displayed in Figure 6.12. Temperatures remained within normal limits until 8 dpi, when two rabbits had temperatures of 40.1°C. These two rabbits also developed a small secondary lesion the following day, when temperatures returned to normal. At 13 dpi, three rabbits had temperatures of 40°C, although temperatures also returned to below 40°C the following day.

Overall, the clinical symptoms following inoculation with 50 pfu of TKO were indistinguishable from those following inoculation with 5000 pfu, except for the slight delay in the appearance of a reaction at the inoculation site.

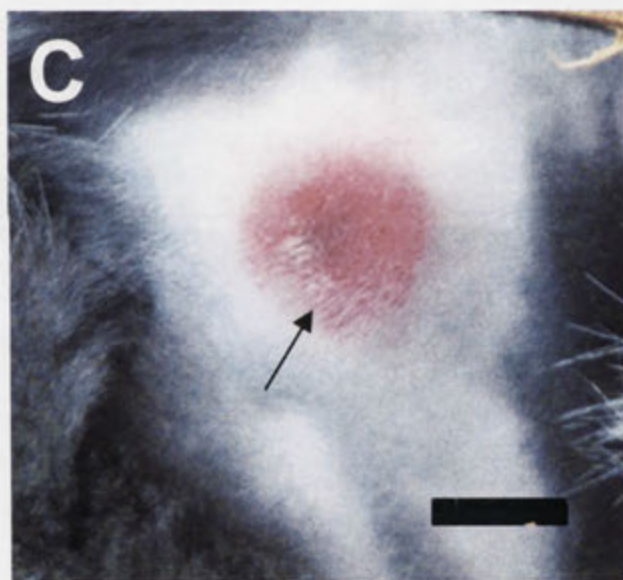
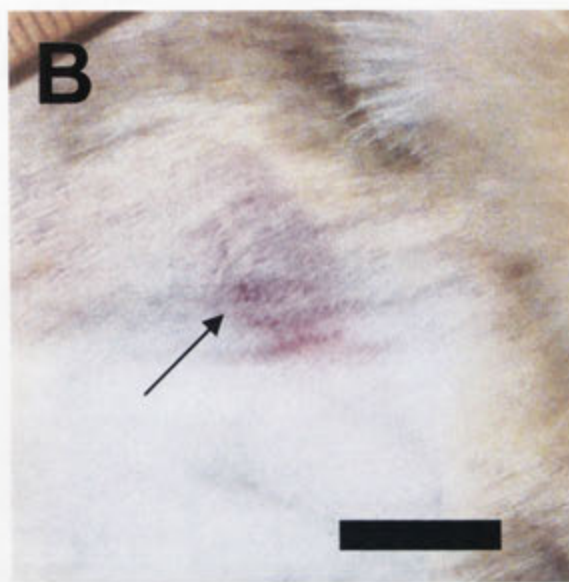
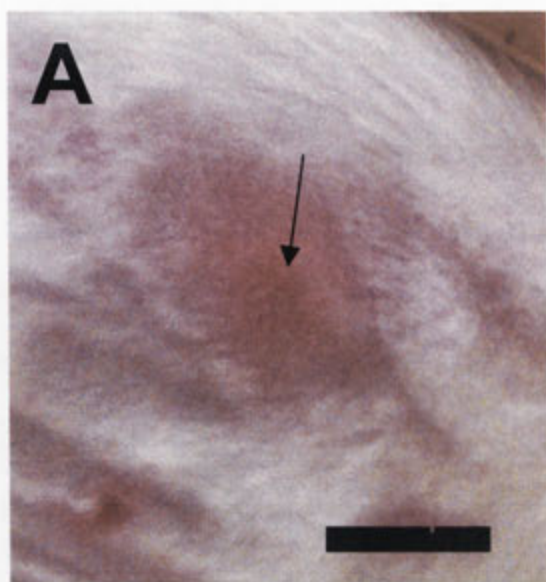
#### *6.2.2.2 SLS challenge of rabbits vaccinated with 50 pfu of TKO at 12 weeks of age*

Six weeks after vaccination, rabbits were challenged with 1000 pfu of SLS on the right thigh and examined for 8 days following infection. The symptoms following challenge with SLS were similar to those exhibited by rabbits that had been vaccinated with 5000 pfu of TKO. Twenty-four hours after challenge, a pink or red area was observed at the inoculation site, which developed into an 8-25 mm red, raised lesion by 3 dpc (Figure 6.13). A single small secondary lesion was noted on the ear of one rabbit (247) at 3 dpc. Several lesions also had scabby material on the surface or displayed dark haemorrhagic patches (Figure 6.13C). By 6 dpc, the lesions at the inoculation site were circumscribed and covered in a hard black scab (Figure 6.14). One animal developed a large lesion at the inoculation site that grew to 35 mm in diameter (Figure 6.14B). This animal did not develop any secondary lesions. The secondary lesion on rabbit 247 was a 5 mm hard pink lump (Figure 6.14E). Animals recovered quickly and by 10 dpc, the only remaining evidence of infection was a scab at the inoculation site. All animals survived challenge with SLS. Rectal temperatures were monitored following challenge and are shown in

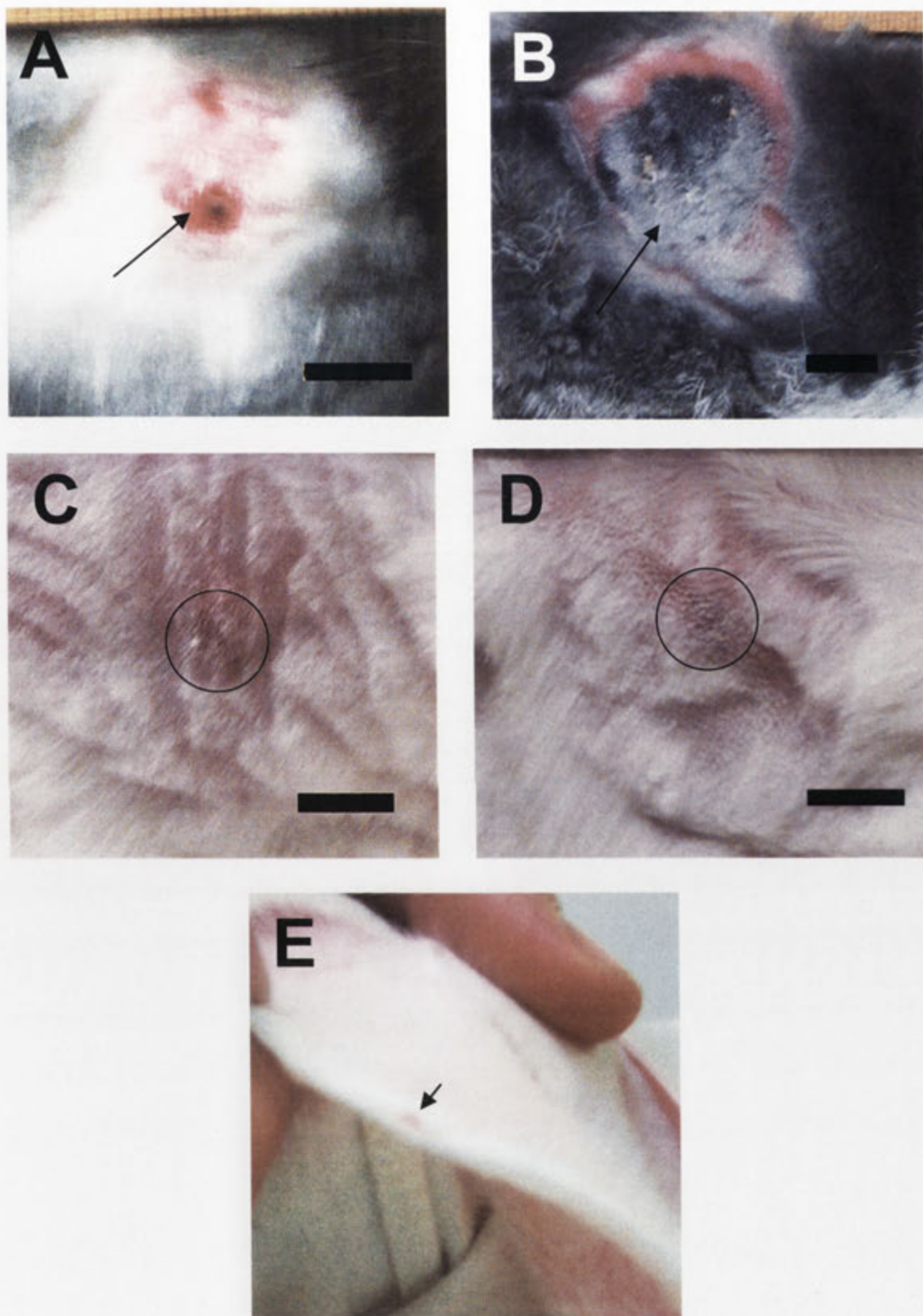


**Figure 6.12** - Rectal temperatures of 12 week old rabbits following vaccination with 50 pfu of TKO.





**Figure 6.13** - Challenge inoculation site of rabbits vaccinated with 50 pfu of TKO at 12 weeks of age, 3 days following challenge with SLS. Representative red, protuberant lesions are shown (A-C, arrowed). Bars on images are 10 mm in size.



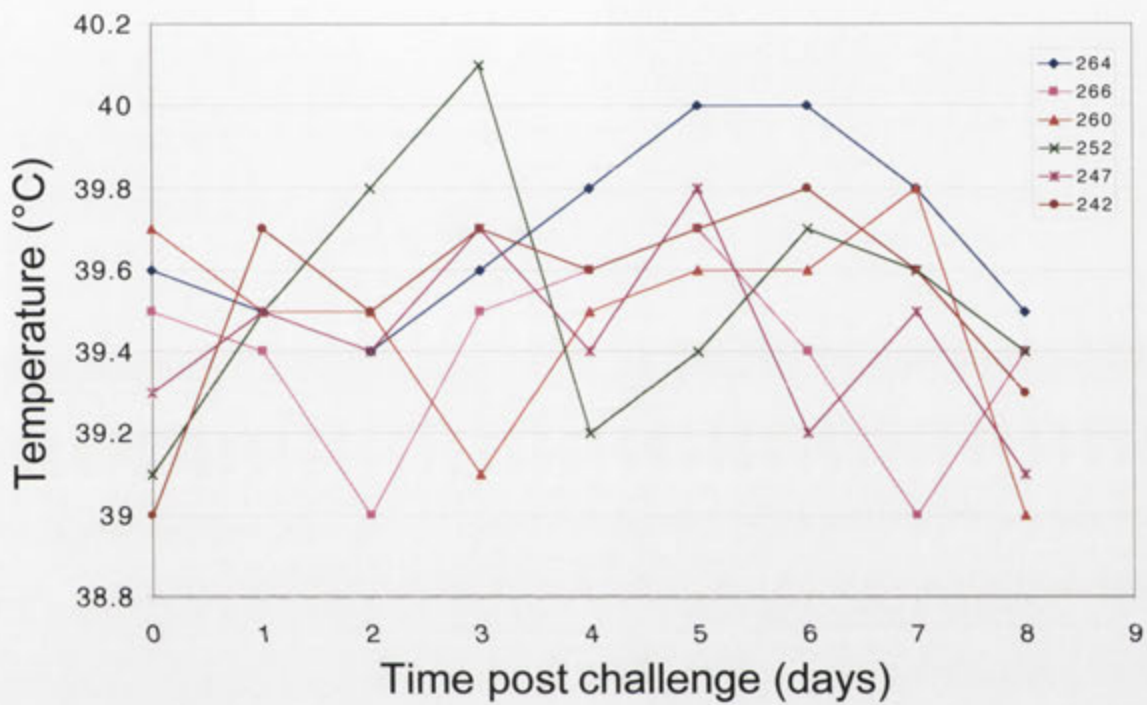
**Figure 6.14 - Primary and secondary lesions of rabbits vaccinated with 50 pfu of TKO at 12 weeks of age, 6 days following challenge with SLS.** Lesions at the inoculation site had completely scabbed over as shown (A and B, arrowed) or developed haemorrhagic patches (C and D, circled). Small secondary lesion on the edge of the ear of rabbit 247 (E, arrowed). Bars on images are 10 mm in size.

Figure 6.15. The temperatures of most animals remained within normal limits, with two animals displaying temperatures of over 40°C for one or two days only.

#### 6.2.2.3 Analysis of anti-myxoma virus antibody responses

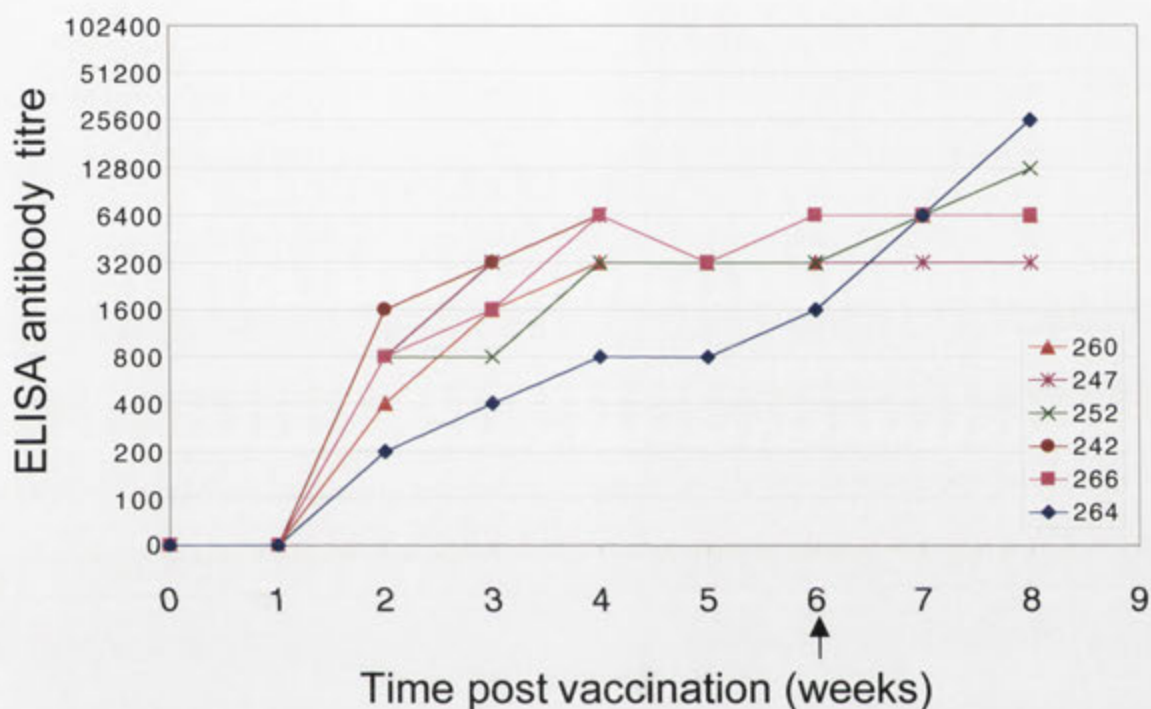
Anti-myxoma virus antibody was first detected in these rabbits at 2 wpv (Figure 6.16). There was no difference in the kinetics of antibody development in 12 week old rabbits given 50 or 5000 pfu of TKO, and by 6 wpv, all rabbits had ELISA antibody titres of between 1600 and 6400. At this time, PRNA titres were also comparable with rabbits that had received 5000 pfu of TKO (Figure 6.8), with 50% neutralisation titres of between 320 and 1280, except for rabbit 264 that had a titre of only 40. This rabbit also had the lowest ELISA titre, although it was only 2-fold lower than three other animals in the group (Figure 6.16).

In the 2 weeks following challenge, only two of the rabbits (252 and 264) showed significant (4-fold or greater) increases in anti-myxoma virus ELISA titres, at 4- and 16-fold respectively (Figure 6.16). These animals were the only ones that had temperatures above 40°C following challenge (Figure 6.15), and rabbit 264 had developed the largest lesion at the inoculation site (Figure 6.14B). However, rabbit 247, which was the only animal in this group to develop a secondary lesion, did not show an increase in ELISA antibody titre (Figure 6.16). The only correlation between ELISA and PRNA titre changes after challenge was shown by rabbit 264, which showed a 128-fold increase in its PRNA titre (Figure 6.15), as well as a 16-fold increase in its ELISA titre. Rabbit 260 was the only other rabbit with a 4-fold or greater increase in PRNA titre but only had a 2-fold increase in ELISA titre following challenge (Figures 6.15 and 6.16).

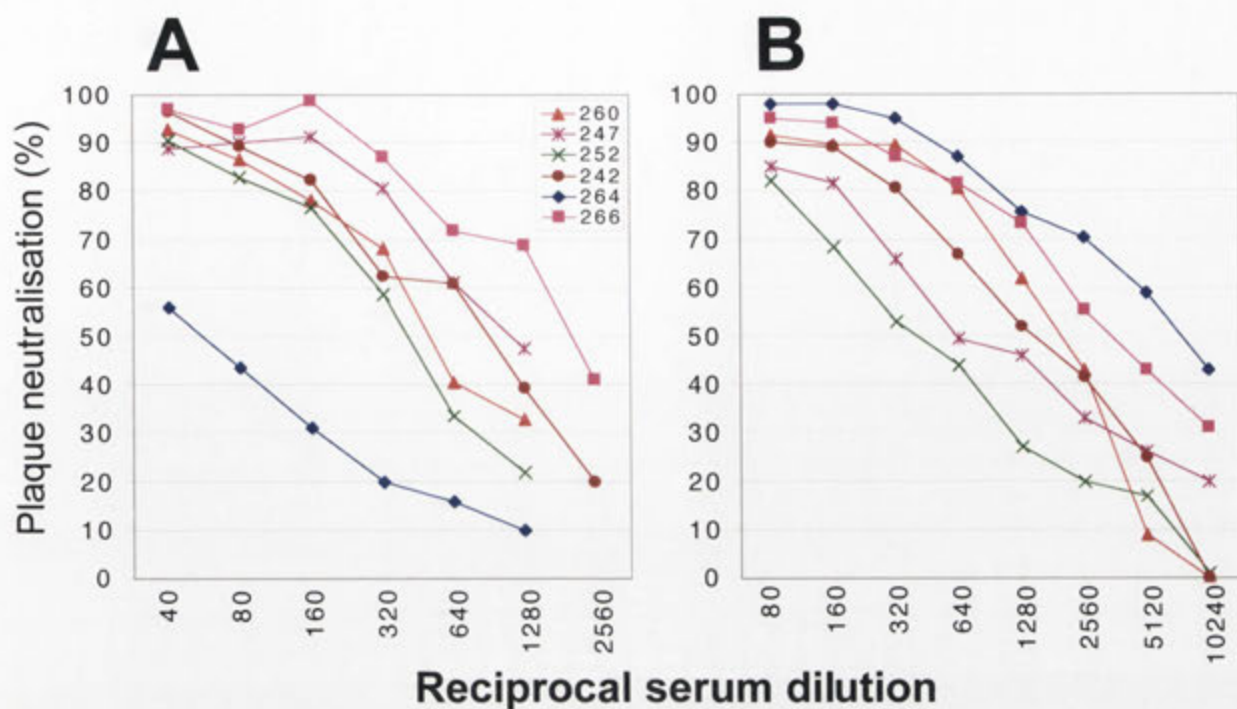


**Figure 6.15** - Rectal temperatures of rabbits vaccinated with 50 pfu of TKO at 12 weeks of age, following challenge with 1000 pfu of SLS.





**Figure 6.16** - ELISA anti-myxoma virus antibody titres in rabbits vaccinated with 50 pfu of TKO at 12 weeks of age. The ELISA titre was determined by titration in duplicate of two-fold serial dilutions of rabbit sera from 1:100. The ELISA endpoint was defined as the reciprocal serum dilution 0.1 OD<sub>405</sub> units above the OD<sub>405</sub> of the 1:100 negative control. A titre of zero was recorded when the OD<sub>405</sub> of 1:100 diluted serum was less than 0.1. Rabbits were challenged with SLS following sera collection at week 6 (arrow).



C	Neutralising antibody titre			
	Pre Challenge (Week 6)		Post Challenge (Week 8)	
	50%	80%	50%	80%
Rabbit	50%	80%	50%	80%
260	320	80	1280	320
247	640	320	320	160
252	320	80	320	80
242	640	160	1280	320
264	40	< 40	5120	640
266	1280	320	2560	640

**Figure 6.17 - Analysis of neutralising anti-myxoma virus antibodies in the serum of rabbits vaccinated with 50 pfu of TKO at 12 weeks of age.** Neutralising antibody was measured by plaque reduction neutralisation assay and the percentage plaque neutralisation recorded at week 6 (A) and week 8 (B) for each serum dilution (two-fold from 1:40 to 1:20480). The titre at which 50% or greater or 80% or greater of myxoma virus plaque formation was neutralised is summarised (C).

### 6.2.3 Testing of TKO in 8 week old rabbits

#### 6.2.3.1 Vaccination of 8 week old rabbits with 50 pfu of TKO

Since 50 pfu appeared to be an effective dose of TKO in 12 week old rabbits, the acceptability of TKO in 8 week old rabbits was evaluated using this lower dose, to determine if rabbits of this age showed more serious vaccine-induced disease. Six 8 week old male domestic rabbits were inoculated with 50 pfu of TKO on the left thigh, and examined daily for 16 days. A summary of the clinical signs of these animals is recorded in Table 6.3. Following inoculation, three rabbits displayed a reaction at the inoculation site at 3 dpi, and two rabbits at 5 dpi, initially taking the form of a reddening of the skin which developed into a small, pink raised lesion within 2 days.

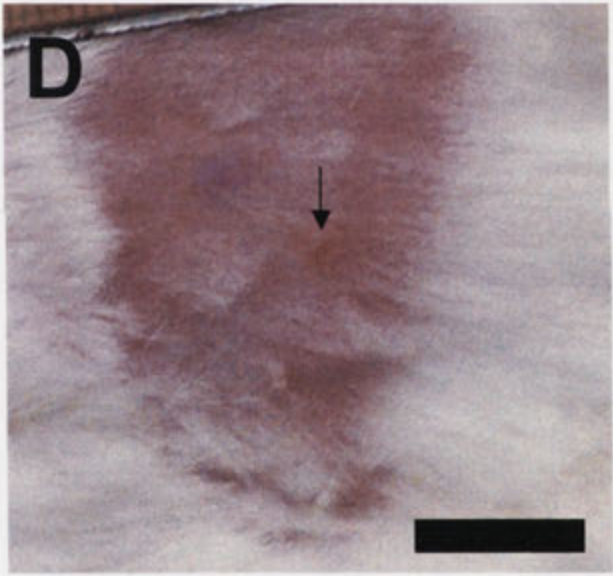
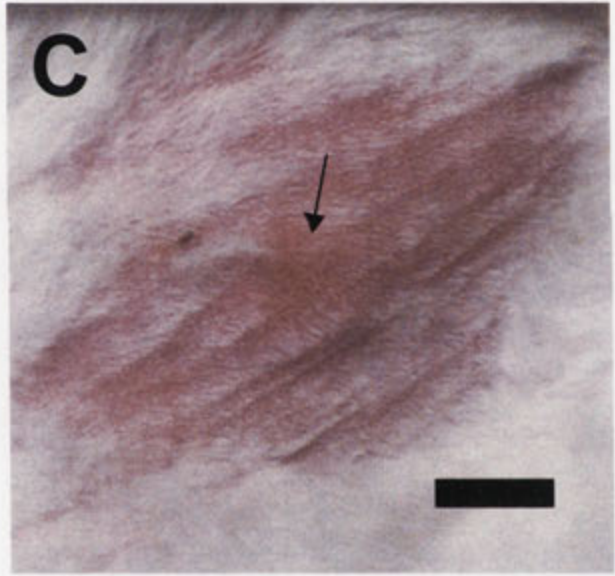
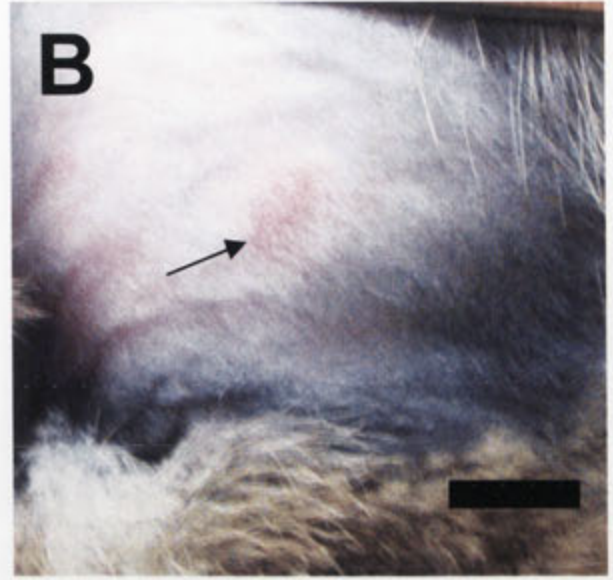
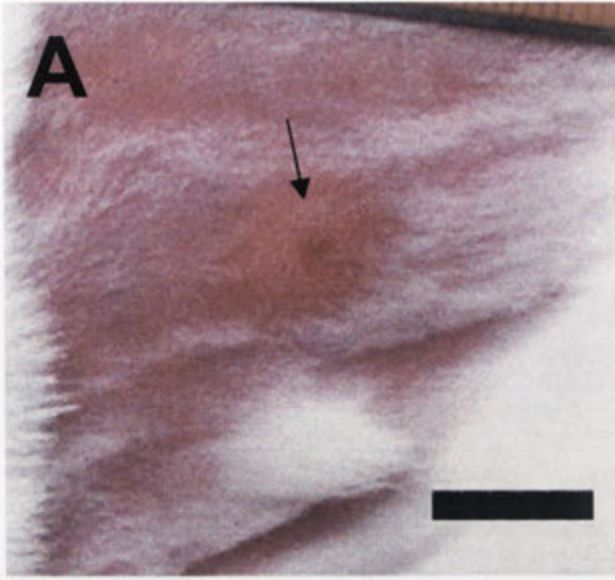
The sixth animal in the treatment group (rabbit 256) displayed very little reaction to the vaccine with no response noted at the inoculation site until 6 dpi (Figure 6.18D). Figure 6.19C shows the inoculation site at 9 dpi. This was negligible, with an 8 mm flat pink lump forming at the inoculation site by 12 dpi (Figure 6.20C), which regressed by 14 dpi. During this time, rabbit 256 did not appear to eat much food and lost weight. The animal was most ill and lethargic at 13 dpi, when its temperature was recorded at 38.4°C (Figure 6.21). This illness could not be attributed to the vaccine virus infection, as clinical signs of myxomatosis were minimal. The rabbit quickly recovered by 15 dpi, although it was of a lower weight than the other animals in the treatment group.

At 6 dpi, the primary lesions of the other five rabbits were pink or red raised lumps less than 10 mm in diameter (three are shown in Figure 6.18A-C) and one rabbit had begun to develop very small secondary lesions on the ear. Eight days following vaccination, these five rabbits had very small secondary lesions evident on the ears or nose. Figure 6.19D and



**Table 6.3** – Clinical signs of 8 week old domestic rabbits following vaccination with 50 pfu of TKO.

Day post inoculation	Clinical signs
3	Slight reddening of skin at the inoculation site
5	Primary lesions of 5/6 rabbits were 8-10 mm in diameter, slightly raised and pink or red in colour. Rabbit 256 had no visible reaction at the inoculation site.
7	Primary lesions were 10-25 mm diameter, red in colour and protuberant. Primary lesion of rabbit 256 was 5 mm in diameter and flat. Several small, red secondary lesions developing on ear of rabbit 243.
8	Primary lesions were 10-20 mm in diameter, protuberant, pink or red in colour. Secondary lesions had developed on ears, nose or eyelids of 5/6 rabbits. Rabbit 256 had only a very small primary lesion (5 mm) and no secondary lesions
10	Primary lesions had haemorrhagic patches, or were covered with a pale flaky scab. Secondary lesions were circumscribed, hard and red. Some had begun to scab over.
14	Primary lesions covered with hard black or brown scab. Secondary lesions were very hard and had begun to regress and scab over. Rabbit 256 had only developed a 10 mm, flat red lesion that did not scab over and regressed by 20 dpi. Rabbit 256 did not develop any secondary lesions.



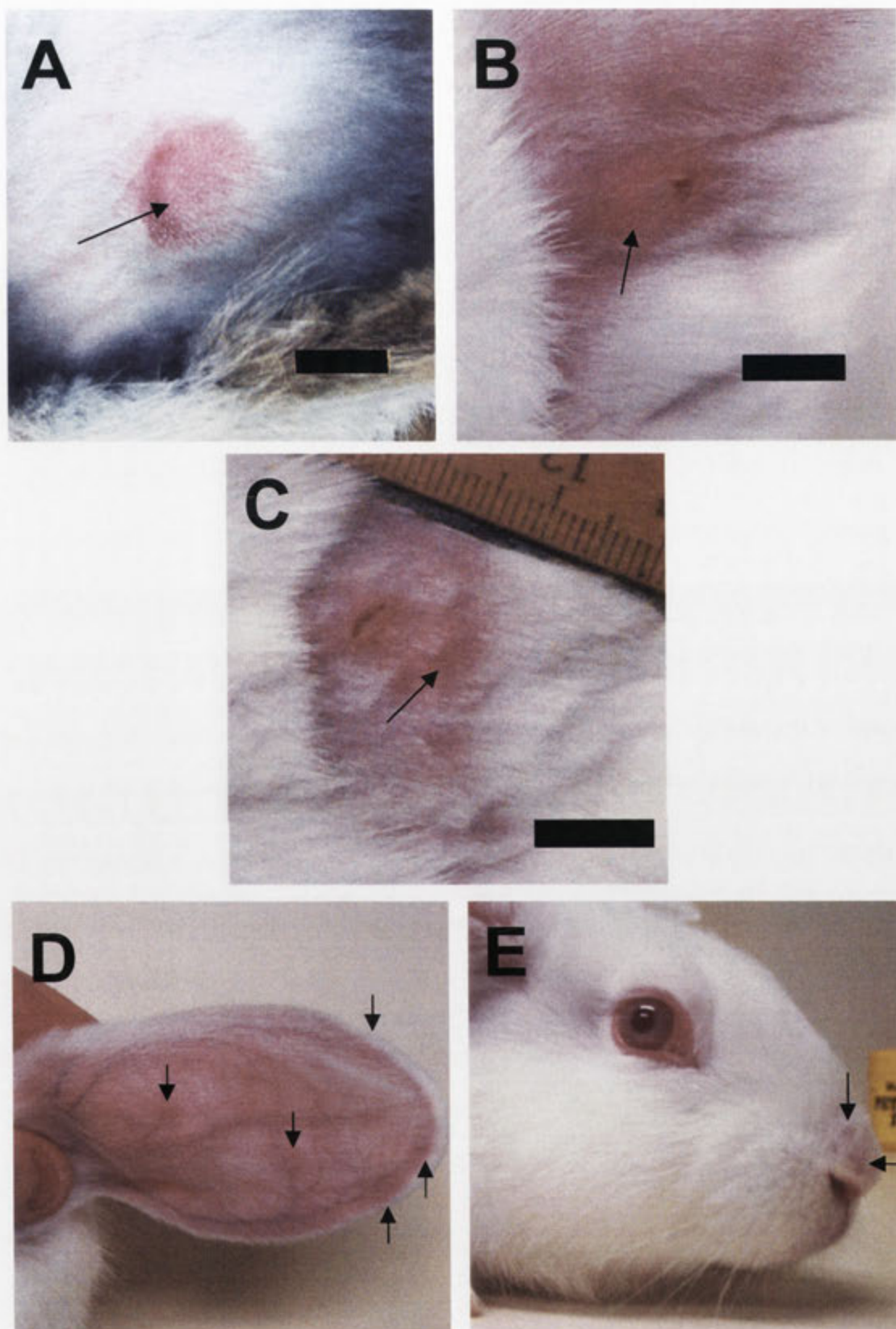
**Figure 6.18** - Inoculation site of rabbits vaccinated with 50 pfu of TKO at 8 weeks of age, 6 days after inoculation. Representative primary lesions of four rabbits (A-D). Reddening of the skin at the inoculation site (A-C, arrowed). Rabbit 256 had a very slight reaction at the inoculation site (D, arrowed). Bars on images are 10 mm in size.

E shows some of these lesions at 9 dpi. One rabbit (250) had at least 10 small lesions (3-6 mm) on the ears, primarily on the edges by 9 dpi (Figure 6.19D). At this time, the primary lesions were circumscribed, 10-15 mm in diameter and several displayed haemorrhagic patches (Figure 6.19A-B). By 12 dpi, primary lesions had scabbed over and begun to regress (Figure 6.20A-B). Secondary lesions on the ears were still evident as small, hard lumps, and several had begun to scab over (Figure 6.20D-E).

Rectal temperatures of rabbits were recorded daily for each animal and are shown in Figure 6.21. Temperatures remained within normal limits ( $<40^{\circ}\text{C}$ ) until 7 dpi, when four of six rabbits had temperatures of  $40^{\circ}\text{C}$  or greater. One rabbit (250) had a temperature of  $40.7^{\circ}\text{C}$  at this point. This rabbit also had the greatest number of secondary lesions (Figure 6.19D). By 9 dpi, temperatures had returned to normal in all rabbits except for rabbit 256.

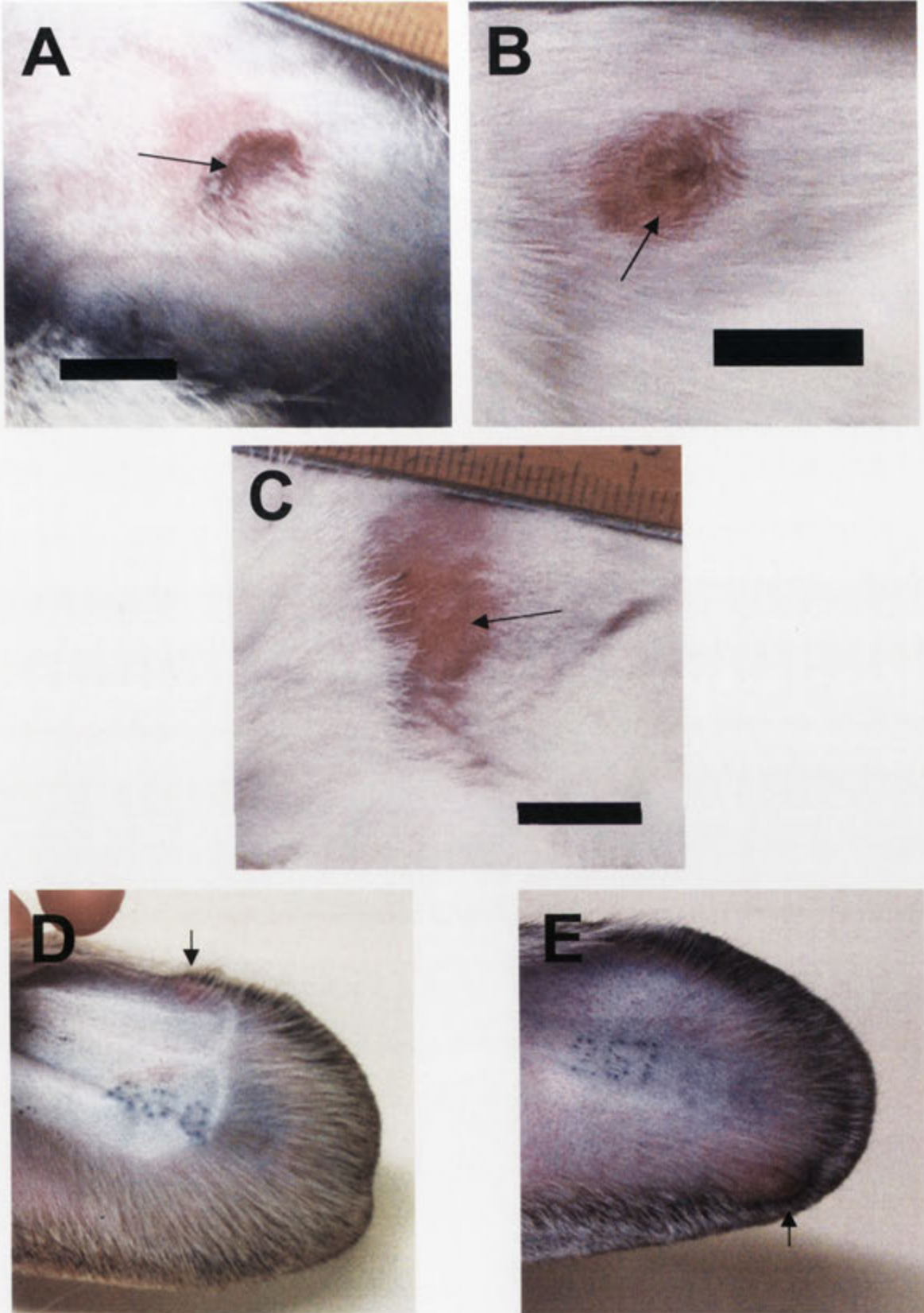
#### *6.2.3.2 SLS challenge of rabbits vaccinated with 50 pfu of TKO at 8 weeks of age*

Six weeks after vaccination, rabbits were challenged with 1000 pfu of SLS on the right thigh and examined daily for 9 days following infection. Within 24 hours of challenge, a 5-8 mm red patch was seen at the inoculation site, which by 3 dpc had developed into a red, protuberant lesion, 9-20 mm in diameter. Several lesions had darker, haemorrhagic patches (Figure 6.22). The lesions had become circumscribed by 6 dpc and most were covered with a hard black scab (Figure 6.23). The skin surrounding the lesions was less inflamed, and by 10 dpc, rabbits had completely recovered from challenge with only residual scab material remaining at the inoculation site. No secondary lesions were noted on any animal following challenge, and all animals survived challenge. The rectal temperatures of rabbits following challenge are shown in Figure 6.24. Rabbit 255 had a slightly higher temperature between 2 and 5 dpc, with a temperature of  $40.1^{\circ}\text{C}$  at 5 dpc. This animal also had the

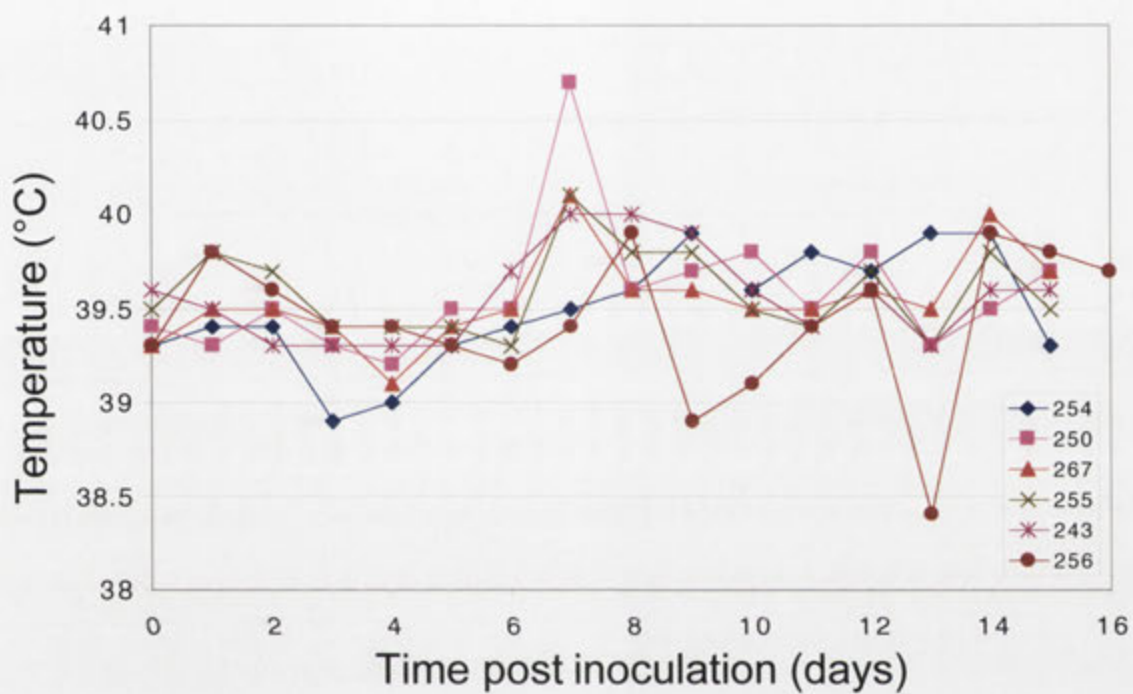


**Figure 6.19** - Primary and secondary lesions on rabbits vaccinated with 50 pfu of TKO at 8 weeks of age, 9 days after inoculation. Primary lesions of three rabbits are shown (A-C, arrowed). On two rabbits, the primary lesion was 10-20 mm in diameter, red and protuberant (A and B, arrowed), with one having a haemorrhagic patch (A, arrowed). There was a slight reaction at the inoculation site on rabbit 256 (C). Secondary lesions on the ear (D, arrowed) and nose (E, arrowed). Bars on images are 10 mm in size.

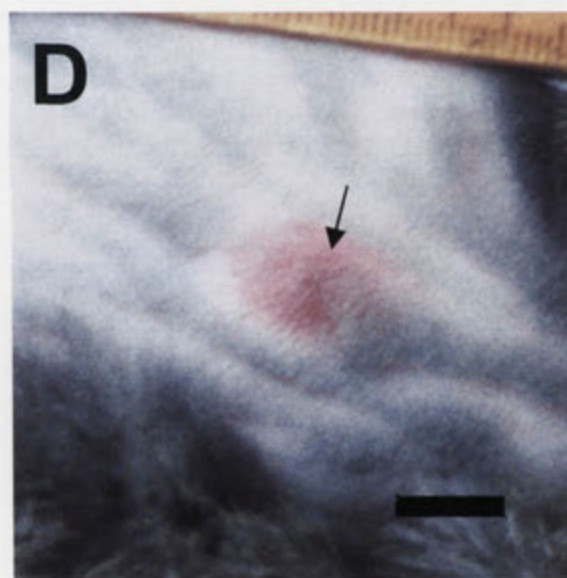
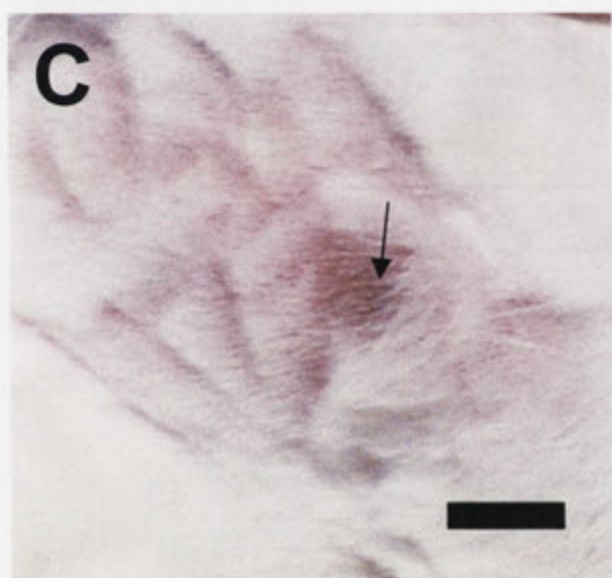
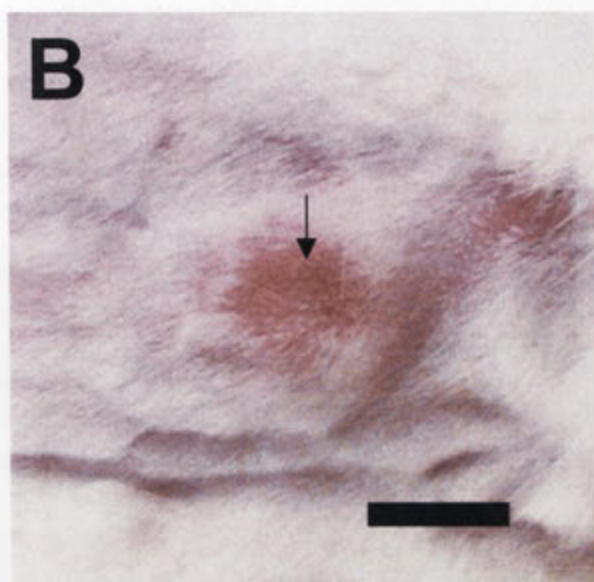
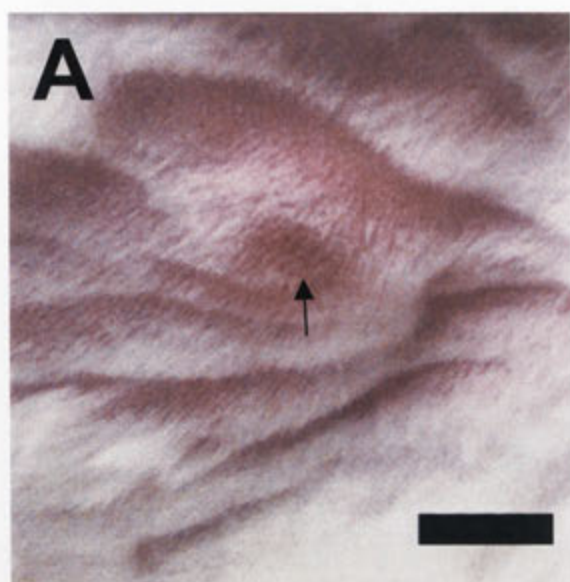




**Figure 6.20** - Primary and secondary lesions on rabbits vaccinated with 50 pfu of TKO at 8 weeks of age, 12 days after inoculation. Representative primary lesions of five rabbits (A and B, arrowed) were 10-20 mm in diameter, and scabbed over as shown. The primary lesion of rabbit 256 was small, flat and red as shown (C, arrowed). Small secondary lesions on the margins of the ears of two rabbits are shown (D and E, arrowed). Bars on images are 10 mm in size.

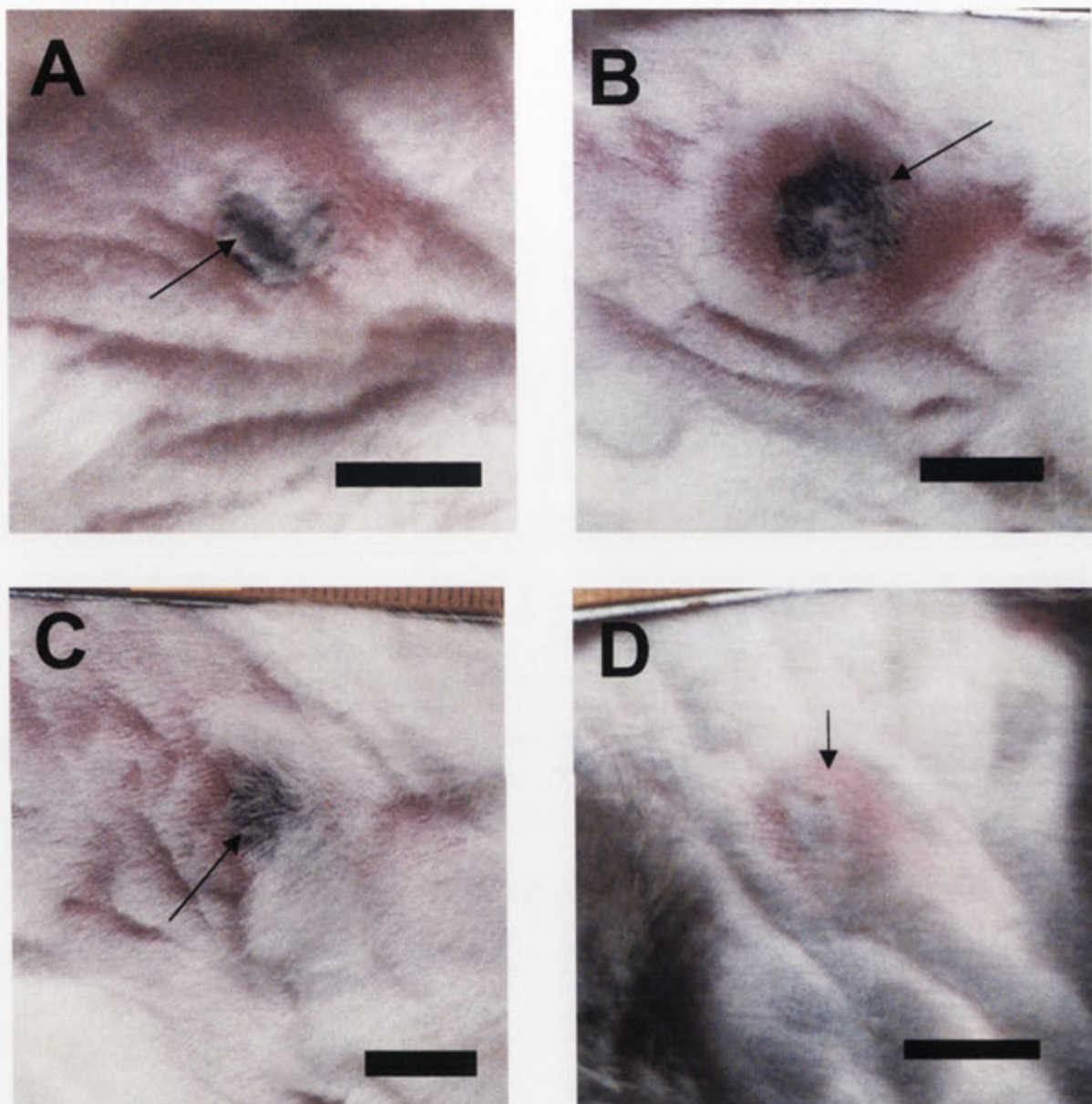


**Figure 6.21** - Rectal temperatures of 8 week old rabbits following vaccination with 50 pfu of TKO.

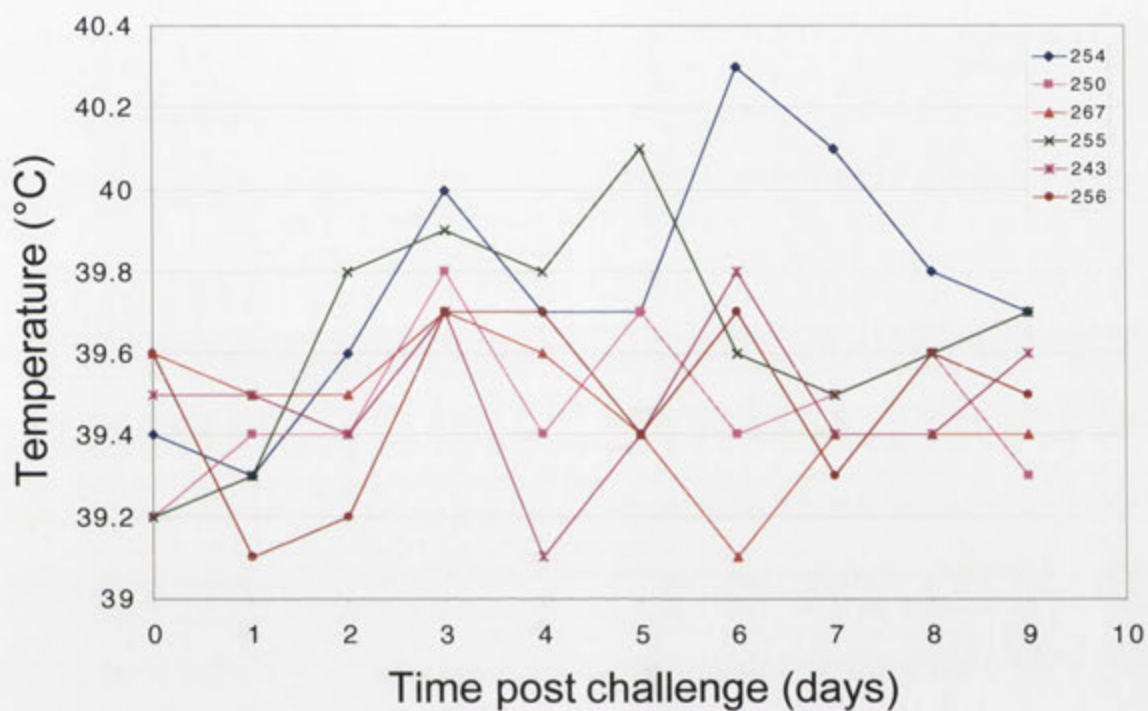


**Figure 6.22** - Challenge inoculation site of rabbits vaccinated with 50 pfu of TKO at 8 weeks of age, 3 days following challenge with SLS. Red, protuberant lesions at the challenge inoculation site as shown (A-D, arrowed), some with haemorrhagic patches (A and C, arrowed). Bars on images are 10 mm in size.





**Figure 6.23** - Challenge inoculation site of rabbits vaccinated with 50 pfu of TKO at 8 weeks of age, 6 days following challenge with SLS. Lesions at the inoculation site were covered with a hard, black scab as shown (A-D, arrowed). Bars on images are 10 mm in size.



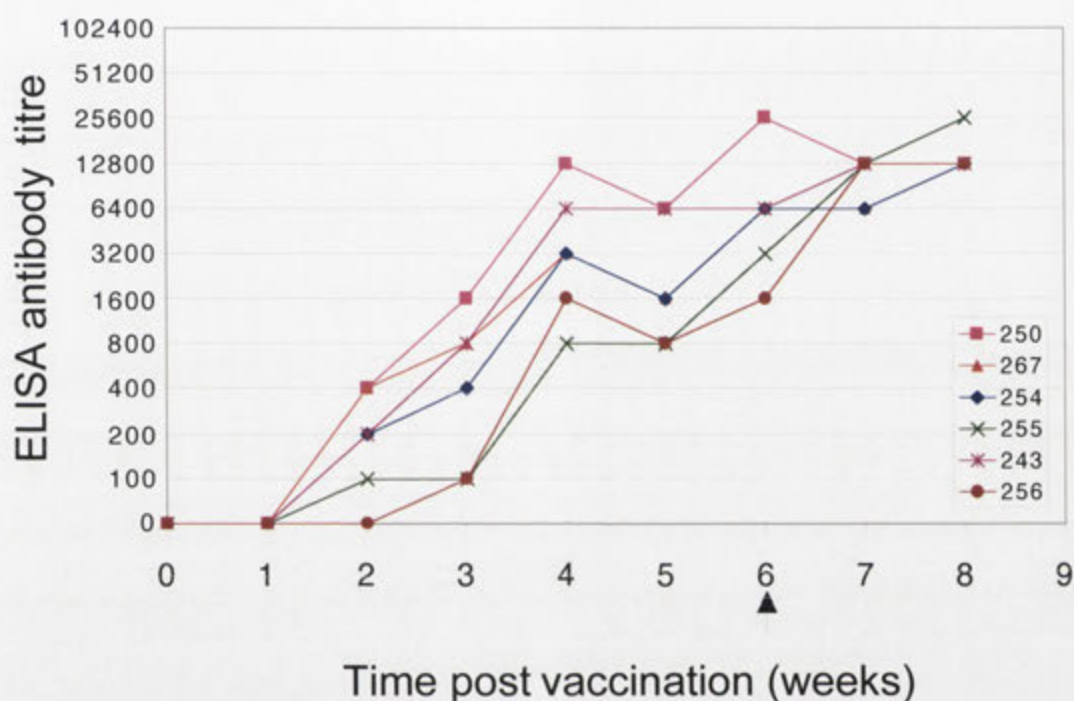
**Figure 6.24** - Rectal temperatures of rabbits vaccinated with 50 pfu of TKO at 8 weeks of age, following challenge with 1000 pfu of SLS.

largest lesion at the inoculation site (Figure 6.23B). Rabbit 254 had temperatures of over 40°C at 3, 6 and 7 dpc. The temperatures of the other four challenged animals remained within normal limits.

#### 6.2.3.3 *Analysis of anti-myxoma virus antibody responses*

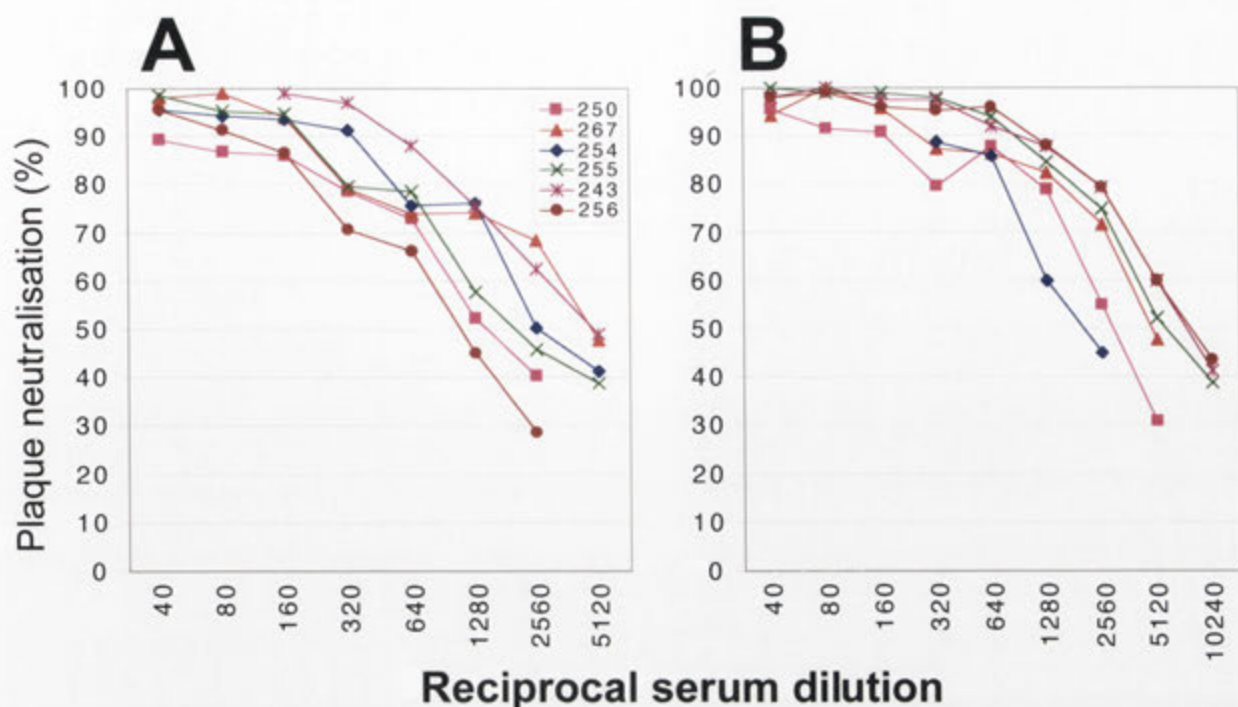
Anti-myxoma virus antibody was first detected by ELISA by 2 wpv, except for rabbit 256, which had exhibited an extremely limited response to the TKO vaccine and did not develop detectable anti-myxoma virus antibodies until 3 wpv (Figure 6.25). However, by the time of challenge at 6 wpv, anti-myxoma virus titres in this rabbit were at 1600, comparable to other rabbits (Figure 6.25). Eight week old rabbits tended to develop anti-myxoma virus antibodies slightly slower than 12 week old rabbits. At 2 wpv, titres ranged between >100 and 400 whilst rabbits vaccinated at 12 weeks old had titres between 200 and 1600 (Figures 6.8 and 6.16). However, by 6 wpv, the range of titres was comparable to other groups of animals, between 1600 and 25600 (Figure 6.25). PRNA titres (50% neutralisation) at this time ranged between 640 and 2560 (Figure 6.26), which was slightly higher (2-4 fold) than that seen in 12 week old rabbits.

In the two weeks following challenge, only two of the six rabbits (255 and 256) showed a significant (8-fold) increase in anti-myxoma virus antibody as measured by ELISA (Figure 6.25). Rabbit 255 had developed the largest lesion upon challenge (Figure 6.23B). These two rabbits were also the only animals to show significant increases in PRNA titres, increasing 4-fold and 8-fold respectively (Figure 6.26). The final ELISA titres of all six rabbits ranged between 12800 and 25600.



**Figure 6.25** - ELISA anti-myxoma virus antibody titres in rabbits vaccinated with 50 pfu of TKO at 8 weeks of age. The ELISA titre was determined by titration in duplicate of two-fold serial dilutions of rabbit sera from 1:100. The ELISA endpoint was defined as the reciprocal serum dilution 0.1 OD<sub>405</sub> units above the OD<sub>405</sub> of the 1:100 negative control. A titre of zero was recorded when the OD<sub>405</sub> of 1:100 diluted serum was less than 0.1. Rabbits were challenged with SLS following sera collection at week 6 (arrow).





**C**

Rabbit	Neutralising antibody titre			
	Pre Challenge (Week 6)		Post Challenge (Week 8)	
	50%	80%	50%	80%
250	1280	160	2560	160
267	2560	160	2560	160
254	1280	320	1280	160
255	1280	160	5120	1280
243	2560	640	5120	1280
256	640	160	5120	1280

**Figure 6.26 - Analysis of neutralising anti-myxoma virus antibodies in the serum of rabbits vaccinated with 50 pfu of TKO at 8 weeks of age.** Neutralising antibody was measured by plaque reduction neutralisation assay and the percentage plaque neutralisation recorded at week 6 (A) and week 8 (B) for each serum dilution (two-fold from 1:40 to 1:10240). The titre at which 50% or greater or 80% or greater of myxoma virus plaque formation was neutralised is summarised (C).

## 6.3 DISCUSSION

### 6.3.1 Acceptability of TKO vaccination in young domestic rabbits

As described in Section 1.6, the ideal myxomatosis vaccine for use in Australia would have the following three characteristics: it should cause no illness or distress in vaccinated animals (acceptability), confer complete protection from challenge with myxoma virus for the lifetime of the animal (efficacy) and be unable to transmit from a recently vaccinated rabbit to others either by direct contact or via arthropod vectors (non-transmissibility).

Although TKO was well tolerated in mature rabbits, there was an increase in the appearance of very small secondary lesions when the virus was administered to 8 or 12 weeks old rabbits. These lesions were smaller and shorter lived than those observed on mature rabbits vaccinated with Uriarra  $\Delta M10L/M11Lgpt$  or Uriarra  $\Delta M-T7gpt$ . Although a cause of little apparent distress to the animals themselves, this may be unacceptable to the owners of rabbits and there is also the potential of long-term scarring at the sites of secondary lesions. Inoculation with a 100-fold lower dose of the vaccine had little effect on the pathology of TKO infection in 12 week old rabbits, except for a slight delay (2-3 days) in the appearance of a reaction at the inoculation site. The incidence of secondary lesions was comparable between animals that received 5000 or 50 pfu of TKO, with three and four rabbits in each group, respectively, developing secondary signs of disease.

These results are comparable to those obtained with other attenuated myxoma virus vaccines. Previous vaccine trials and field trials have shown an increased incidence of vaccine-induced disease in younger rabbits, including those vaccinated with the French SG33 strain (Saurat *et al.*, 1978; Vautherot *et al.*, 1997). Similarly, inoculation of newborn rabbits with RFV, which has no ill effects on rabbits greater than 6 weeks of age (Fenner

and Woodroffe, 1954; Marlier *et al.*, 2000), has resulted in high levels of morbidity and mortality including generalised disease (Duran-Reynals, 1945; Hyde and Gardner, 1939). However, amongst the 18 young animals vaccinated in this study, none developed any more serious signs of disease than several small secondary lesions, and there was no evidence of generalised immunosuppression in rabbits as young as 8 weeks old.

Although TKO induced some very small secondary lesions in 8 and 12 week old rabbits, it did confer complete protection from myxoma virus challenge at 6 weeks after vaccination. All TKO-vaccinated rabbits developed a lesion at the inoculation site, a higher proportion than found amongst mature animals (12/18 rabbits). Dissemination of virulent challenge virus within the rabbits was extremely limited, with only two out of 18 animals developing secondary signs of myxomatosis. These were limited to a single, very small, secondary lesion on the eyelid or ear of each. As four of the 18 mature animals vaccinated with 5000 pfu of TKO developed secondary lesions, it seems that there was little difference in the protective efficacy of TKO between mature and younger domestic rabbits.

Another consideration is the potential transmissibility of TKO from younger rabbits. Because the pathology of TKO infection was slightly more severe than that observed in mature rabbits, the virus may exceed transmissible titres for longer periods of time, or may achieve higher titres for longer periods in young wild rabbits, increasing the potential transmissibility of TKO. This would require further investigation including, most importantly, direct insect transmission studies in younger animals to determine the actual risk of TKO escape and establishment in the field.



### 6.3.2 Conclusions

Although TKO was safe and effective in mature rabbits, it induced very mild secondary signs of myxomatosis in younger animals, and may not be suitable for use in rabbits as young as 8-12 weeks old. Also, as demonstrated in Chapter 5, the titre of virus at the inoculation site was above potentially transmissible levels ( $10^7$  pfu/g), at least temporarily in mature rabbits, and in a small proportion of wild animals, and the formal possibility of transmission of TKO from a recently vaccinated domestic rabbit to wild rabbits exists. Together, these factors indicate that TKO would not be an ideal candidate for a widely used, live attenuated myxomatosis vaccine.

## **Chapter 7**

# **Construction and testing of a replication-deficient myxoma virus vaccine**

## CHAPTER 7 – CONSTRUCTION AND TESTING OF A REPLICATION-DEFICIENT MYXOMA VIRUS VACCINE

### 7.1 INTRODUCTION

As demonstrated in Chapters 5 and 6, live virus vaccines are able to elicit excellent protective responses in rabbits. However, even the highly attenuated TKO vaccine occasionally induced very small secondary lesions in younger animals, and reached transmissible titres in the skin of some mature domestic rabbits. One approach that would prevent vaccine transmission is to use a replication-deficient virus that was capable of entering host cells and expressing viral antigens following inoculation. In contrast to TKO, it would be unable to assemble new virus particles *in vivo* and increase virus titre to transmissible levels or disseminate to other tissues and induce disease. The production of viral antigens by this virus within host cells, especially professional antigen presenting cells, should allow the induction of a broad range of virus-specific host effector mechanisms, most importantly CTL responses, which are believed to be critical for protection against myxoma virus (Kerr and McFadden, 2002).

As described in Section 1.4.3, the replication-deficient VV strain MVA has proven effective at protecting mice from lethal VV challenge (Wyatt *et al.*, 2004) and monkeys from monkeypox virus challenge (Earl *et al.*, 2004). The use of MVA before administration of a replication-competent VV vaccine also reduced the severity of the lesion induced by the live VV vaccine in monkeys (Earl *et al.*, 2004). However, live replication-deficient vaccines to protect against poxvirus diseases have not been evaluated outside of the Orthopoxvirus models of mouse/VV and monkey/monkeypox virus.

This chapter describes the testing of a replication-deficient myxoma virus vaccine that was constructed through the deletion of the myxoma virus putative host-range gene M063R. The selection of this gene was based on information provided by Professor Grant McFadden (John P. Robarts Research Institute, London, Ontario, Canada - provisional patent application 111702GM-RRI). M063R is a 20 kDa cytoplasmic protein that is expressed early during infection. It is one of three adjacent genes in the myxoma virus genome (M062R, M063R and M064R) which all share some homology with other poxvirus genes including VV C7L and capripoxvirus CF8a. These genes share the conserved Pox\_C7\_F8A domain and are all predicted host-range genes. A strain of VV not expressing C7L has been investigated, which exhibited limited replication in hamster Dede cells, but normal replication in most human cell lines (Oguiura *et al.*, 1993), further suggesting a host-range function for genes with this conserved domain.

Studies using a  $\Delta$ M063R strain of Lausanne virus have been conducted by McFadden *et al.* (personal communications). This virus was constructed by inactivation of M063R by insertion of the *E. coli*  $\beta$ -galactosidase gene in the M063R open reading frame. This virus was able to infect and grow in Vero cells to similar levels as wild type virus. However, the  $\Delta$ M063R virus was unable to replicate in rabbit cell lines including RK13, RL-5, Hig82 (rabbit synovial cells) and SIRC (rabbit corneal fibroblasts). It was determined that expression of late viral genes (as measured by immunoblot analysis of Serp1 expression) was prevented in rabbit cells infected with the  $\Delta$ M063R virus. At a low multiplicity of infection (MOI) of 0.001, expression of the early gene M-T7 or the late gene Serp1 was not detected in infected RK13 cells by immunoblot analysis. At higher MOIs (1.0), a low level of M-T7 expression, but no Serp1 expression was detected. Similarly, when enhanced green fluorescent protein (EGFP) was placed under control of an early myxoma virus

promoter in the  $\Delta$ M063R virus, a low level of EGFP expression was detected in infected RK13 cells. However, when expressed under a synthetic late promoter, no EGFP expression was found (Grant McFadden, personal communication).

The function of M063R in myxoma virus infected cells is unknown, although it may be involved in inhibiting the cellular response to interferons (Grant McFadden, personal communication). Without M063R, myxoma virus may be unable to productively infect rabbit cells due to the induction of interferon-response genes. An abortive infection, apparently specific for rabbit cells is induced (although some early gene expression does occur) due to the cessation of host cell biosynthetic activity. Interestingly, inoculation of rabbits with 1000 pfu of this replication-deficient strain of Lausanne conferred complete protection from challenge with virulent myxoma virus 15 days after vaccination (Grant McFadden, personal communication).

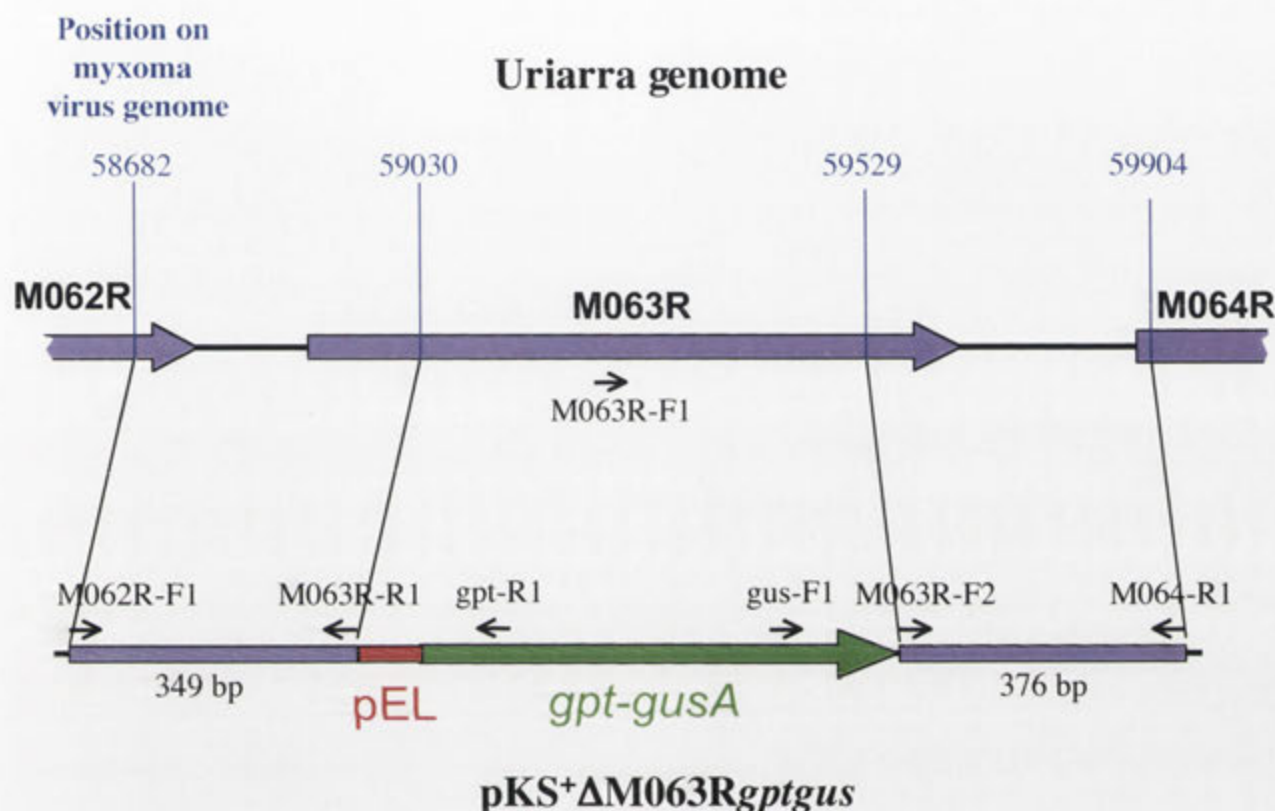
In this chapter, I will describe the construction and testing of a myxoma virus Uriarra strain with a deletion in the M063R open reading frame to evaluate the safety and efficacy of a replication-deficient vaccine using the myxoma virus/rabbit poxvirus disease model. This virus strain was analysed to confirm its replication-deficient nature, and tested for its acceptability and protective efficacy against lethal myxoma virus challenge as a single dose vaccine, and as part of a boosting regimen with either a second dose of the replication-deficient virus or TKO.

## 7.2 RESULTS

### 7.2.1 Design and construction of pKS<sup>+</sup>ΔM063Rgptgus and Uriarra ΔM063Rgptgus

The inactivation of the M063R open reading frame (ORF) was achieved by homologous recombination in Uriarra-infected Vero cells with the plasmid pKS<sup>+</sup>ΔM063Rgptgus (Figure 7.1). This plasmid contains two regions of sequence homologous to the Uriarra genome, between which was cloned a selective marker, the *gpt-gusA* fusion gene (Cao and Upton, 1997) under the control of the synthetic early/late poxvirus promoter (Davidson and Moss, 1989). A recombination event between the Uriarra genomic DNA and the plasmid results in a deletion of 498 bp in the middle of the M063R gene (54.2% of the ORF), and its replacement with the pEL-*gpt-gusA* cassette, allowing for selection of the recombinant virus by resistance to mycophenolic acid, and screening for recombinant plaques by blue staining in the presence of X-gluc (Section 2.5.3.4).

As the recombinant virus was predicted to be unable to productively infect rabbit cells including the RK13 cell line, the initial virus/plasmid recombination was performed in Vero cell monolayers. Isolation of single recombinant viral plaques was conducted by infecting monolayers of the primate cell line BSC-1 with this recombinant virus preparation, and selecting viral plaques that stained blue in the presence of X-gluc. BSC-1 cells were used because plaques were evident on the cell monolayer as clusters of cells rather than the holes in the monolayer that form on Vero cell monolayers. This made staining and picking of isolated recombinant plaques easier. After six rounds of plaque purification, four recombinant virus clones were recovered. To determine if these clones contained any wild type virus, they were examined by PCR. RK13 or Vero cell monolayers were infected with the viruses at an MOI of 0.1 and incubated for 72 hours. Viral DNA was isolated and subjected to two PCR analyses - one pair of primers (M063R-



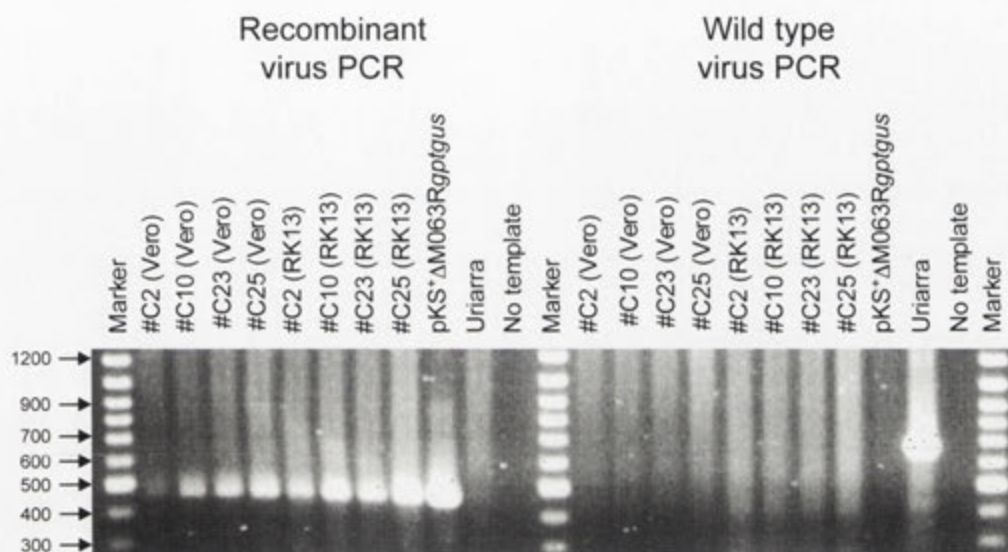
**Figure 7.1** - Construction of plasmid for deletion of M063R sequences of myxoma virus. Sequences (349 and 376 bp) homologous to the 3' and 5' ends of M063R were cloned by PCR from the genome of Uriarra using the primers indicated (small black arrows, Table 2.3). The *gpt-gusA* fusion gene was cloned between these sequences under poxvirus synthetic early/late promoter (pEL) control. Homologous recombination of this plasmid with the Uriarra genome results in a recombinant virus (Uriarra ΔM063R*gptgus*) with a 498 bp deletion in the middle of M063R. The positions of other primers used for the PCR screening of recombinant viruses (M063R-F1, gpt-R1 and gus-F1) are indicated. The position of the ends of the homologous sequences are indicated as described in the published myxoma virus sequence, Genbank AF170726. Figure is not to scale.



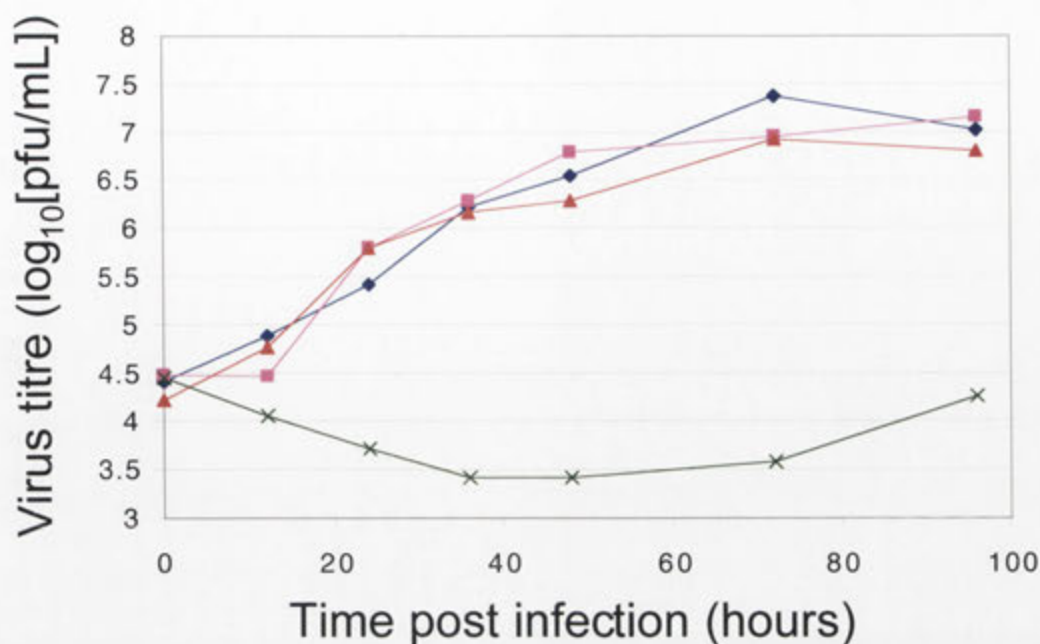
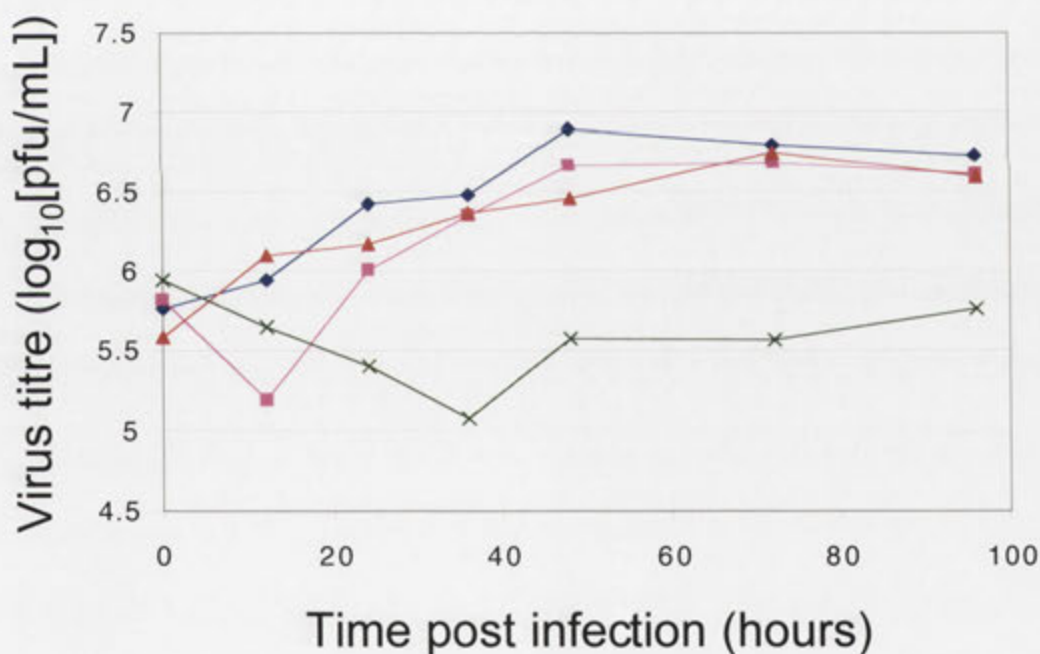
F1 x M064R-R1) was used to amplify wild type DNA only, whilst a second pair (M062R-F1 x gpt-R1) was used to amplify recombinant viral DNA only. Only recombinant virus PCR product (440 bp) was amplified from all four virus preparations (Figure 7.2). The infection of RK13 cell monolayers was included, as these cells would have preferentially amplified wild type virus that was capable of replication in rabbit cell lines. Isolate #C10 was selected for further analysis and named Uriarra  $\Delta$ M063Rgptgus. Cloning and sequencing of the M063R locus showed sequence to be the same as that of the plasmid pKS<sup>+</sup> $\Delta$ M063Rgptgus, indicating that a homologous recombination event between the plasmid and Uriarra genomic DNA had resulted in the loss of 54.2% of the M063R ORF.

### **7.2.2 *In vitro* growth characteristics of Uriarra $\Delta$ M063Rgptgus in RK13 and Vero cells**

To compare the growth kinetics of Uriarra (wild type) and Uriarra  $\Delta$ M063Rgptgus, Vero and RK13 cell monolayers were infected with viruses at a MOI of 0.1 or 3.0, and the titre of virus determined at time points following infection (Section 2.5.6). Uriarra and Uriarra  $\Delta$ M063Rgptgus had comparable growth characteristics in Vero cells, increasing in titre to very similar levels (Figure 7.3). However, Uriarra  $\Delta$ M063Rgptgus did not increase in titre over the observed time period when grown in RK13 cells. At a MOI of 0.1, the titre dropped from  $2.9 \times 10^4$  pfu/mL to  $2.6 \times 10^3$  pfu/mL by 48 hours post infection (hpi), and increased slightly to  $1.85 \times 10^4$  pfu/mL by 96 hpi, whilst Uriarra titres increased to  $2.4 \times 10^7$  pfu/mL by 72 hpi (Figure 7.3A). A similar pattern was observed at a MOI of 3.0, where Uriarra and Uriarra  $\Delta$ M063Rgptgus exhibited very similar growth patterns in Vero cells. However, whilst Uriarra grown in RK13 cells increased from  $3.9 \times 10^5$  pfu/mL to  $5.68 \times 10^6$  pfu/mL by 72 hpi, Uriarra  $\Delta$ M063Rgptgus decreased in titre to  $1.2 \times 10^5$  pfu/mL by 36 hpi and remained lower than original titres during the course of the experiment (Figure 7.3B).



**Figure 7.2 - PCR analysis of Uriarra  $\Delta$ M063Rgptgus.** DNA was isolated from four clones of Uriarra  $\Delta$ M063Rgptgus (C2, C10, C23 and C25) that had been grown for 3 days on Vero or RK13 cell monolayers and analysed by PCR to identify the presence of recombinant virus or wild type virus. The plasmid pKS<sup>+</sup>ΔM063Rgptgus was used as a positive control for recombinant virus, and Uriarra genomic DNA was used as a positive control for wild type virus. A recombinant virus PCR product (440 bp) was amplified from all virus preparations except Uriarra. Wild type PCR product (660 bp) was amplified from the Uriarra control only.

**A****B**

**Figure 7.3 - Growth of Uriarra and Uriarra  $\Delta$ M063Rgptgus in Vero and RK13 cell monolayers.** Vero or RK13 cell monolayers were infected with Uriarra or Uriarra  $\Delta$ M063Rgptgus at an MOI of 0.1 (A) or 3.0 (B), and the titre of virus in duplicate samples was determined up to 96 hours post infection.

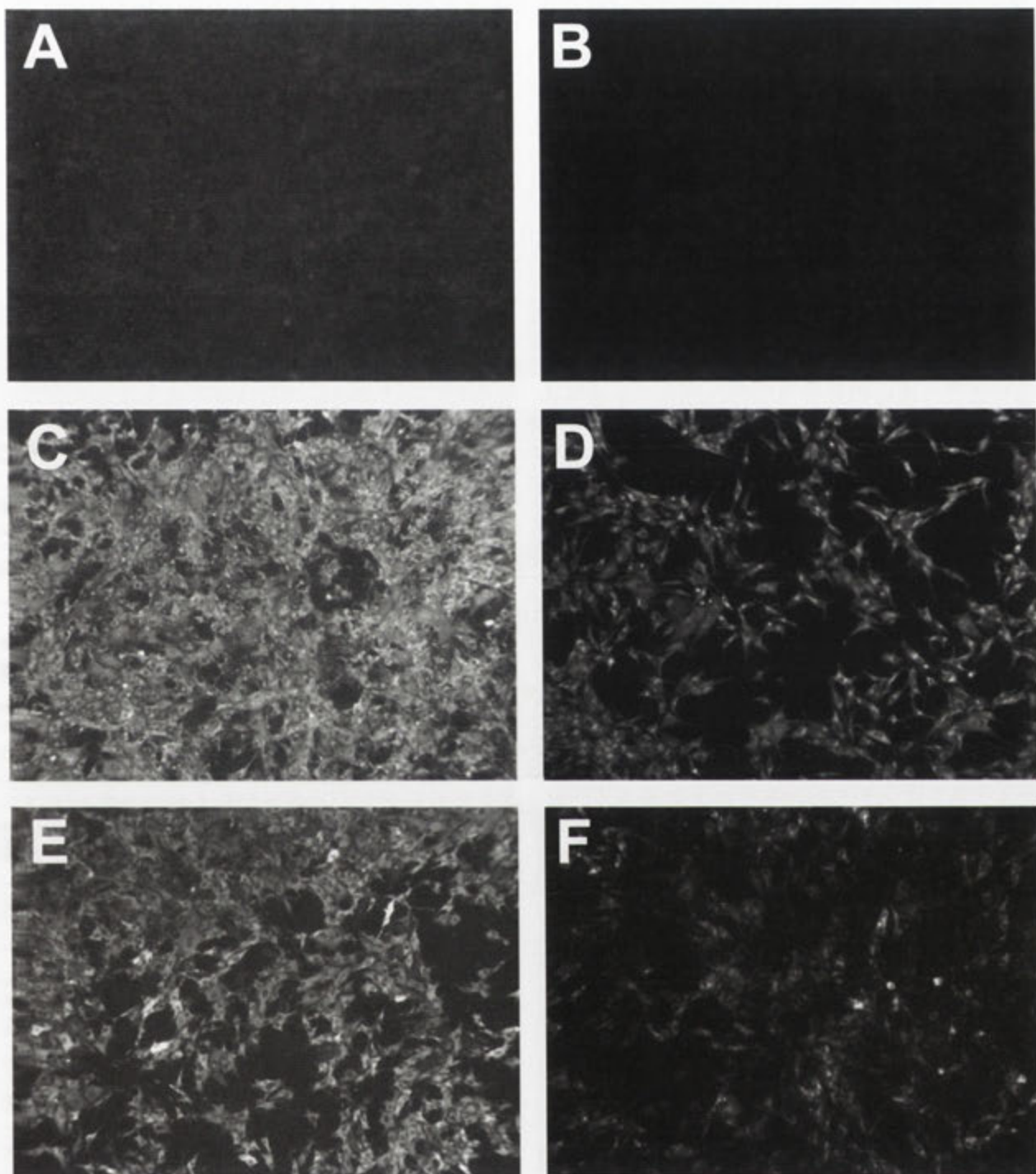
Key:  $\blacklozenge$  - Uriarra in Vero;  $\blacksquare$  - Uriarra  $\Delta$ M063Rgptgus in Vero;  $\blacktriangle$  - Uriarra in RK13;  $\times$  - Uriarra  $\Delta$ M063Rgptgus in RK13.

These data suggest that Uriarra  $\Delta$ M063Rgptgus is unable to replicate in RK13 cells *in vitro*, although replication in Vero cells was very similar to its parental virus, Uriarra.

### 7.2.3 Expression of intermediate/late genes in Uriarra $\Delta$ M063Rgptgus-infected RK13 and Vero cells

Monolayers of Vero or RK13 cells were infected with Uriarra or Uriarra  $\Delta$ M063Rgptgus at a MOI of 1 and incubated for 24 hours before fixing with methanol:acetone. The monolayers were probed with the monoclonal antibody 3B6E4, specific for an unknown intermediate or late-expressed myxoma virus protein, the expression of which is inhibited if viral DNA replication is inhibited (Peter Kerr, personal communication). The staining of infected Vero cell monolayers appeared similar regardless of the infecting virus, with intensely fluorescent cells observed across the monolayer (Figure 7.4C and E). Uriarra-infected RK13 cell monolayers also exhibited a high level of fluorescence, with almost all cells on the monolayer possessing an intense fluorescence throughout the cytoplasm (Figure 7.4D), although fluorescence intensity appeared lower than in infected Vero cells (Figure 7.4C). However, Uriarra  $\Delta$ M063Rgptgus-infected RK13 cell monolayers exhibited a reduced fluorescence intensity, with most cells staining poorly with a patchy appearance, and with approximately half of the cells on the monolayer staining no more intensely (Figure 7.4F) than uninfected control cells (Figure 7.4B).

These data suggests that the intermediate/late myxoma virus protein is being expressed in at least some Uriarra  $\Delta$ M063Rgptgus-infected RK13 cells, although the amount of protein produced is generally less than in Vero cells or RK13 cells infected with Uriarra, and many cells do not appear to express the protein.



**Figure 7.4 - Immunofluorescent detection of myxoma virus antigen in infected cells.** Vero cell monolayers were mock infected (A), infected with Uriarra (C) or Uriarra  $\Delta$ M063Rgptgus (E) at an MOI of 1. RK13 cell monolayers were mock infected (B), infected with Uriarra (D) or Uriarra  $\Delta$ M063Rgptgus (F) at an MOI of 1. After 24 hours, cells were fixed and probed with the monoclonal antibody 3B6E4, specific for an intermediate or late myxoma virus antigen. All photos were taken at 40X magnification.

## **7.2.4 Vaccination of rabbits with Uriarra $\Delta$ M063Rgptgus and protective efficacy against SLS challenge**

### *7.2.4.1 Vaccination of rabbits*

Six domestic rabbits of 10-13 months of age were vaccinated with 100,000 pfu of Uriarra  $\Delta$ M063Rgptgus intradermally on the left thigh, and examined daily for 2 weeks following vaccination. A summary of the clinical signs of rabbits is recorded in Table 7.1. There was no reaction at the inoculation site of most rabbits (Figure 7.5A-D), apart from a slight pinkness of the skin of one rabbit (Figure 7.5E), until 5 days post inoculation (dpi), when a small pink lump formed on all rabbits, ranging in size from 3-5 mm. By 7 dpi, this lump was slightly protuberant, pink or red in colour and up to 15 mm in diameter (Figure 7.6). The appearance of the lesion had not changed significantly over the next 7 days except for a reduction in size and a brown scab forming over it on two of the rabbits. By 14 dpi, the lesion was a smaller (<10 mm) hard lump, which had faded in colour to pink (Figure 7.7). By 21 dpi, the lesion had resolved completely on all rabbits. There were no secondary lesions or other signs of disease observed on any animal following vaccination. The rectal temperatures of all animals remained within normal limits (38.9-39.9°C) over the 14 days during which temperatures were monitored (Figure 7.8).

### *7.2.4.2 Challenge of rabbits with SLS*

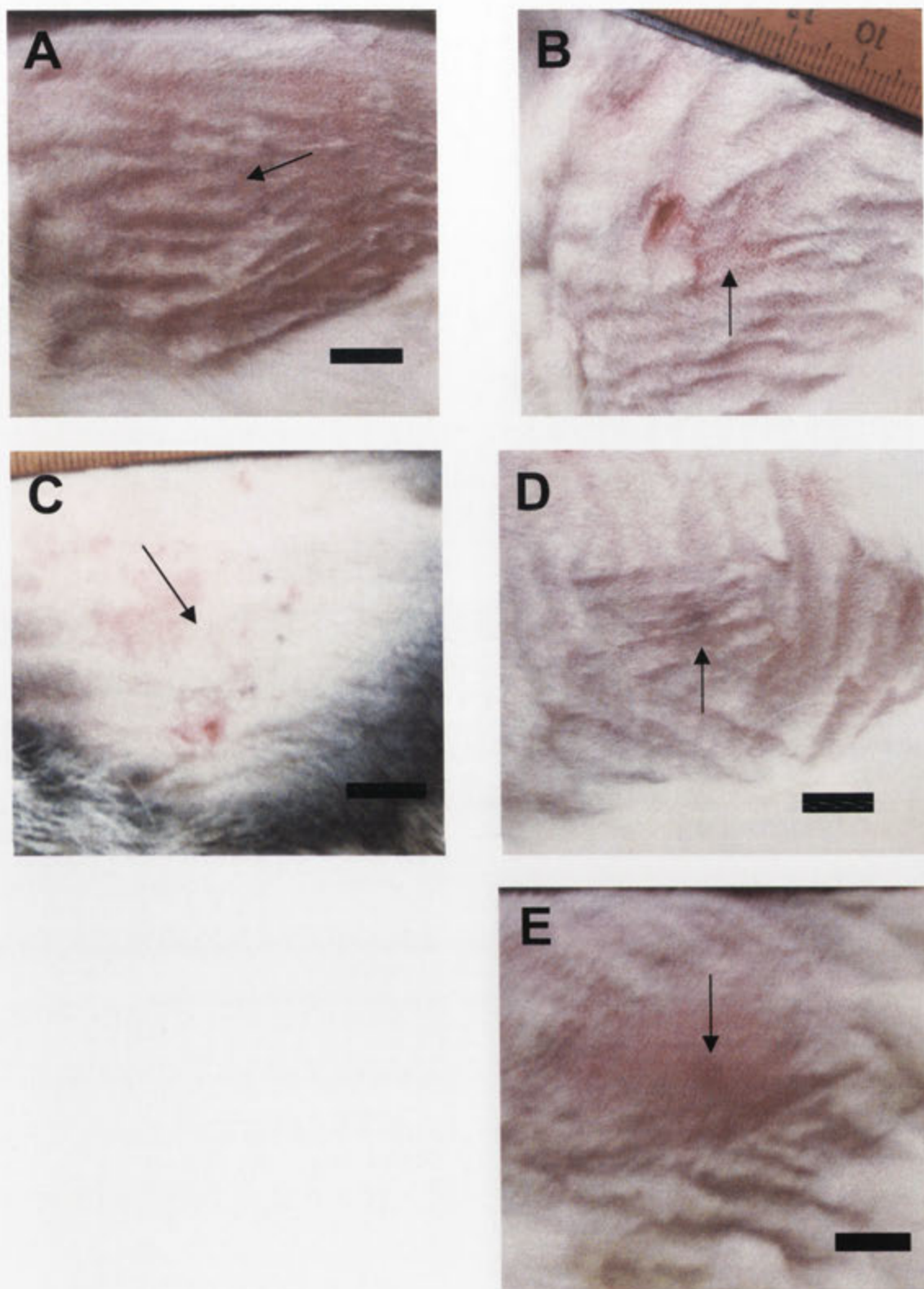
Six weeks after vaccination, all Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits were challenged with 1000 pfu of SLS intradermally on the left thigh, and examined for signs of disease for 14 days. The clinical signs of rabbits following challenge are summarised in Table 7.2. Within 24 hours of challenge, a pink lump was seen at the inoculation site, ranging in size from 8-12 mm in diameter. By 3 days post challenge (dpc), these lesions had increased in size up to 25 mm, and were quite protuberant (Figure 7.9); some lesions were dark crimson



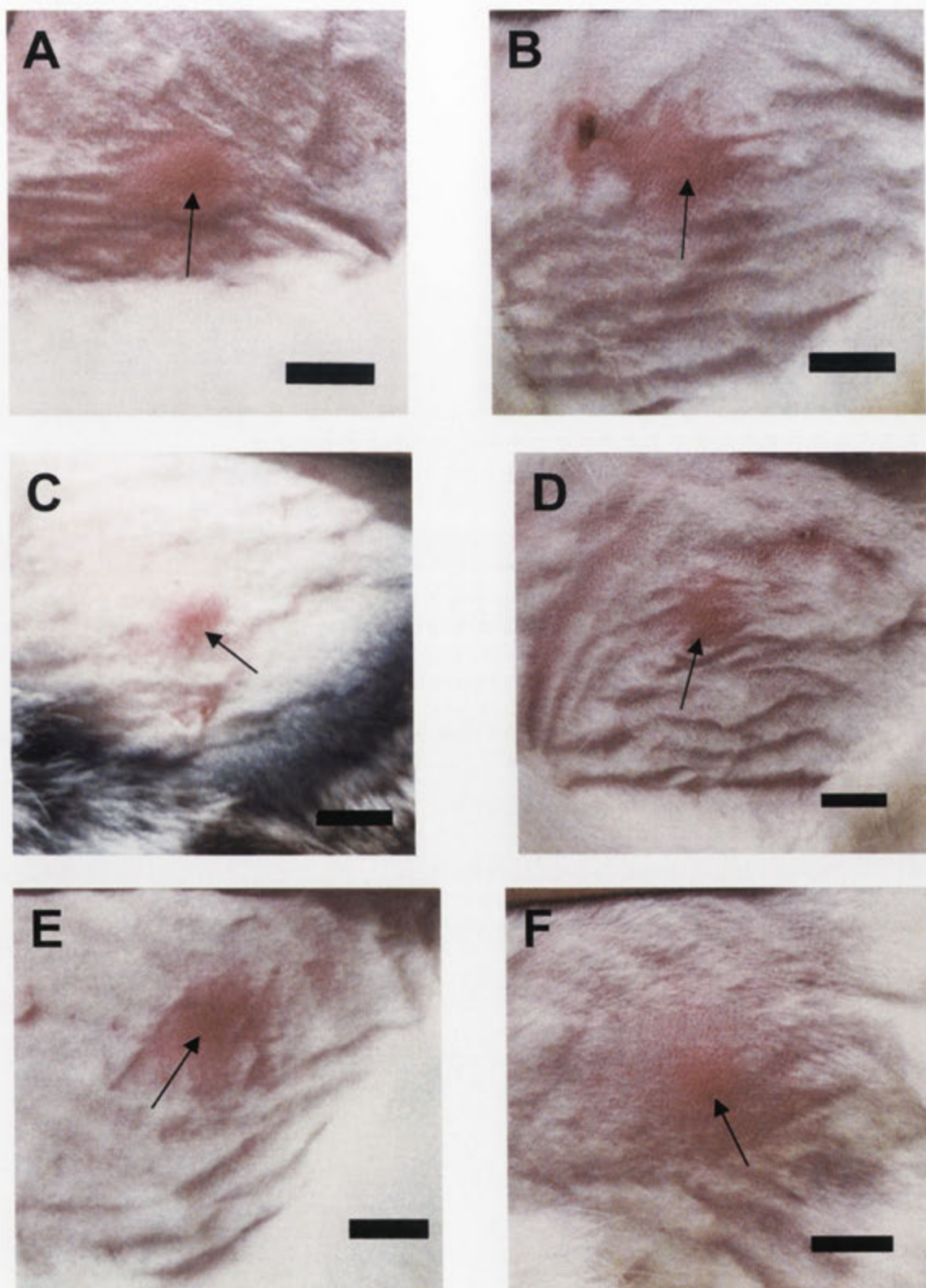
**Table 7.1** – Clinical signs of mature domestic rabbits following vaccination with 100,000 pfu of Uriarra ΔM063R*gptgus*.

Day post inoculation	Clinical signs
2	No reaction at the inoculation site
3	Pinkness to skin at the inoculation site of one rabbit (183)
5	Pink lump (3-5 mm) at inoculation site
7	Red or pink raised lump, 8-15 mm in diameter
14	Hard lump at inoculation site, 8-10 mm. Black or brown scab on 2 animals.
21	No lesion. Slightly darker skin at inoculation site.



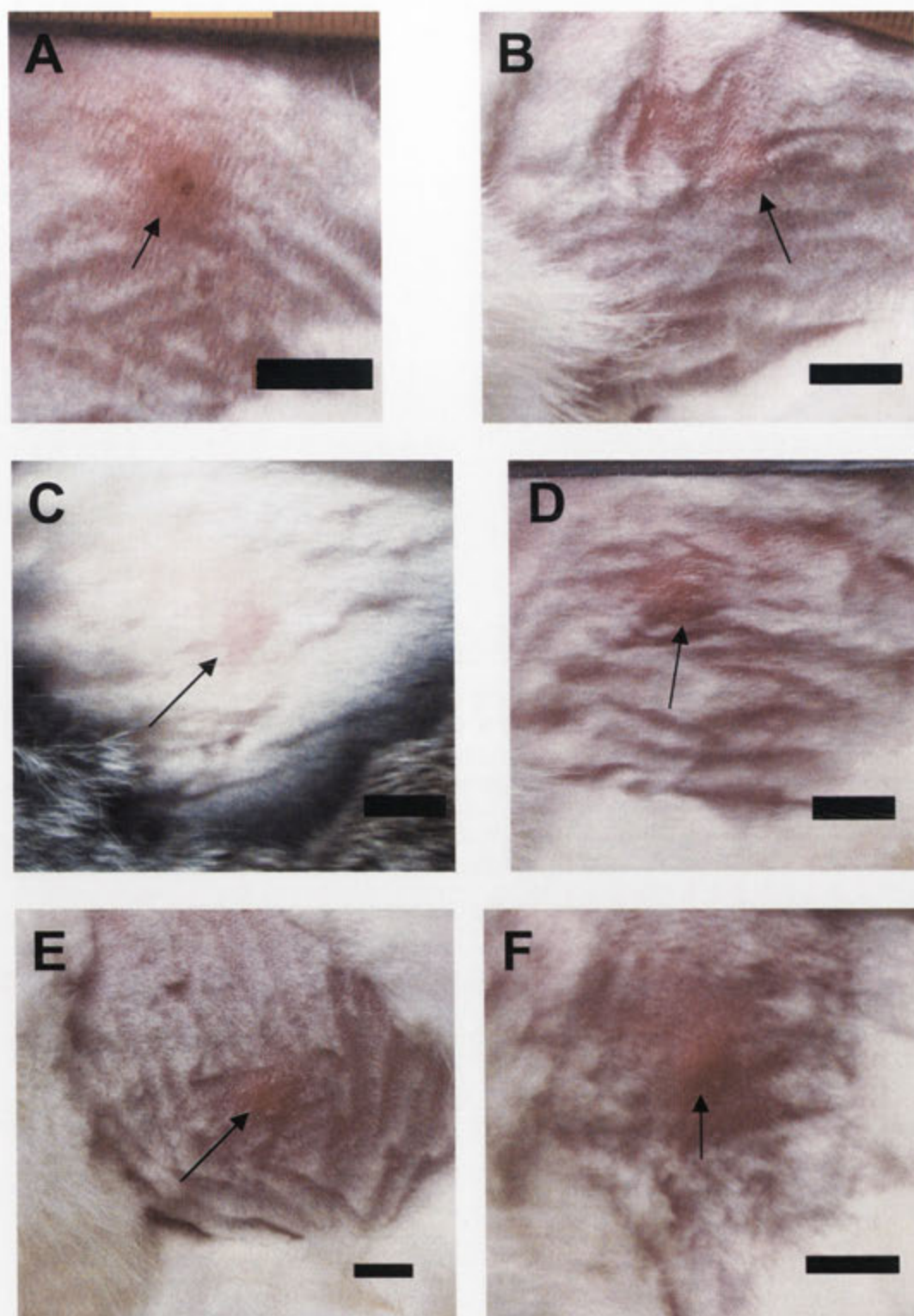


**Figure 7.5 - Inoculation site of rabbits vaccinated with 100,000 pfu of Uriarra  $\Delta$ M063Rgptgus 3 days after inoculation.** There was no reaction observed at the inoculation site (arrowed) on any rabbit at this time point, except for a slight pinkness on one rabbit (E, arrowed). Each image position corresponds to the same rabbit in Figures 7.6 and 7.7. Bars on images are 10 mm in size.

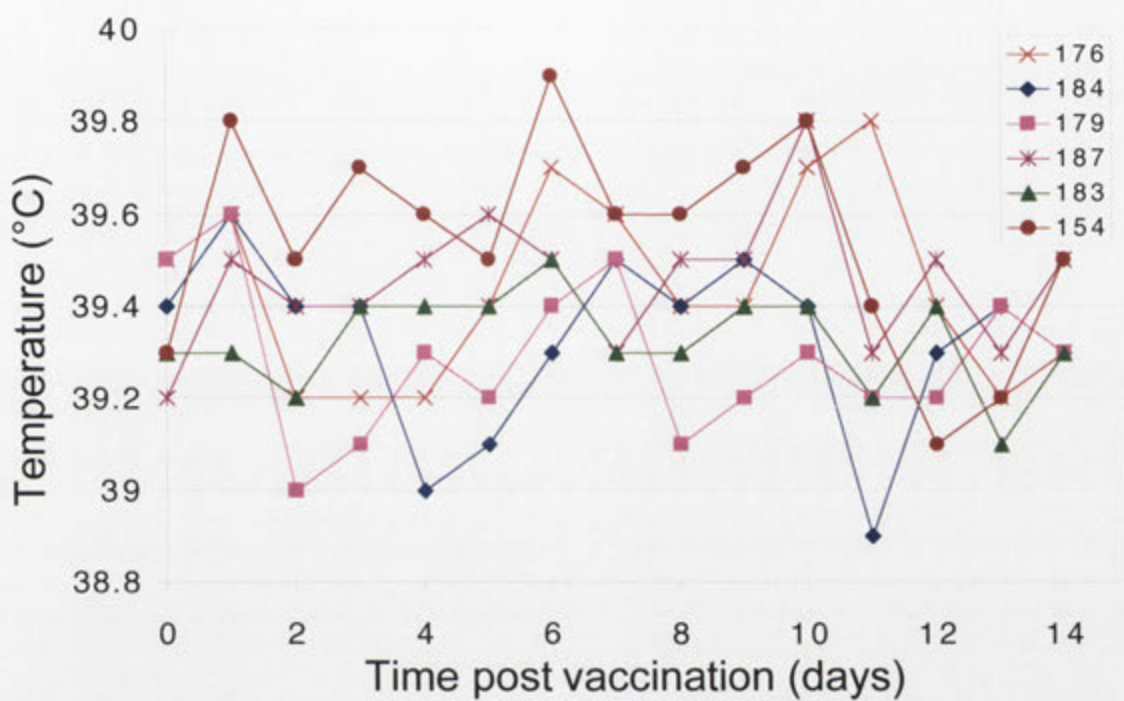


**Figure 7.6** - Primary lesion of rabbits vaccinated with 100,000 pfu of Uriarra  $\Delta$ M063Rgptgus, 7 days after inoculation. The inoculation site of all rabbits had developed a small (5-15 mm) raised pink lump (A-F, arrowed). Bars on images are 10 mm in size.





**Figure 7.7 - Primary lesion of rabbits vaccinated with 100,000 pfu of Uriarra  $\Delta$ M063Rgptgus, 14 days after inoculation.** The primary lesions were raised, pink or red lumps no larger than 10 mm in diameter (A-F, arrowed). Some lesions had scabbed over (A, E). Bars on images are 10 mm in size.

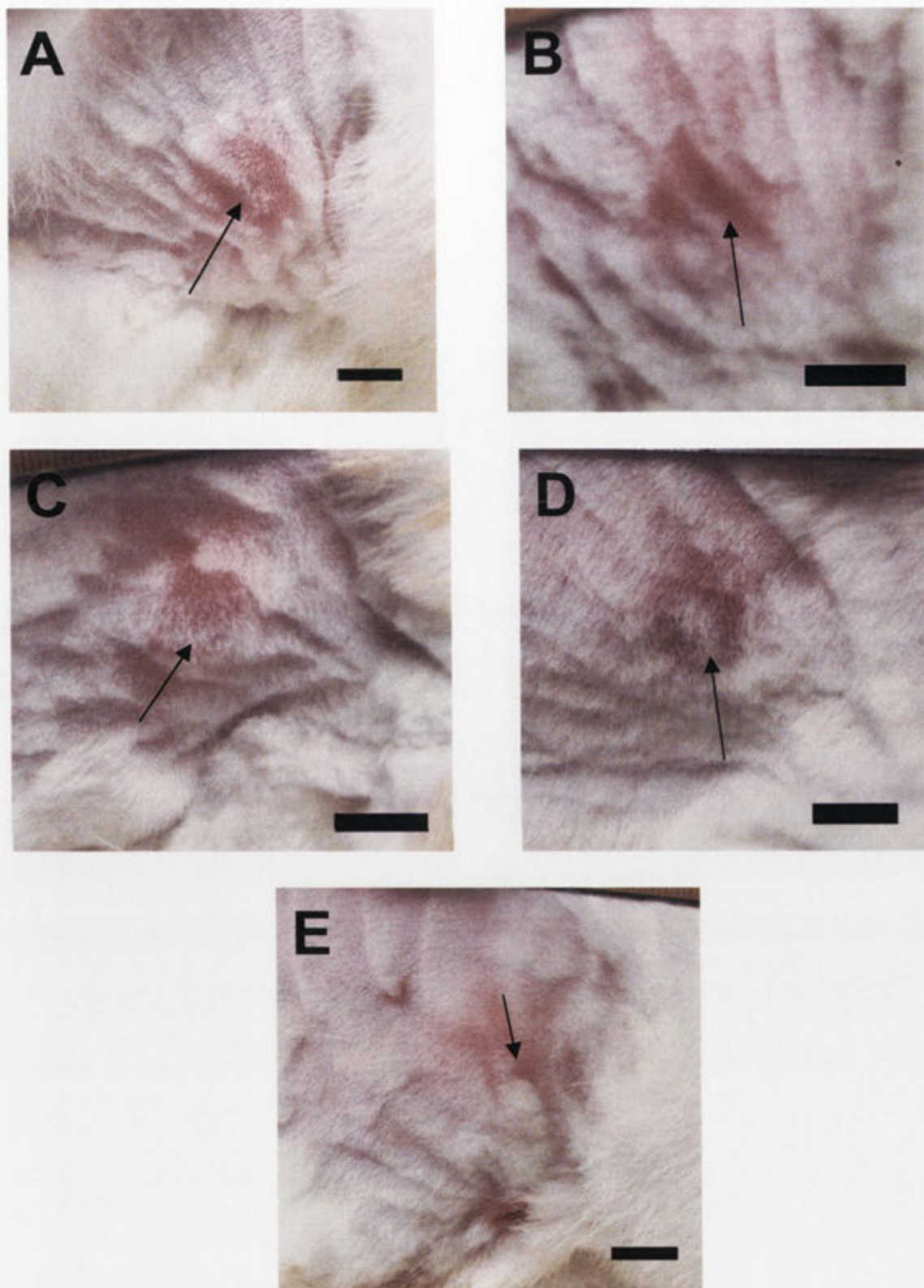


**Figure 7.8** - Rectal temperatures of rabbits following vaccination with 100,000 pfu of Uriarra  $\Delta$ M063Rgptgus.

**Table 7.2** – Clinical signs of Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits following challenge with SLS.

Day post inoculation	Clinical signs
1	Pink lesion at the inoculation site, 8-12 mm diameter.
3	Red or crimson protuberant lesion, 15-25 mm diameter.
5	Lesions at the inoculation site 20-40 mm in diameter, black in colour. Secondary lesions on 5/6 rabbits on eyelids, body, ears and/or face. All animals had a temperature of $>40^{\circ}\text{C}$ .
7	Lesions at the inoculation site were protuberant and covered by a hard black scab, 25-40 mm diameter. More numerous secondary lesions; all had turned black in colour, though still swollen and hard; 5-10 mm in diameter; located on eyelids, ears, anus, scrotum, body, face or nostrils. 4/6 animals had a temperature of $>40^{\circ}\text{C}$ .
8	Rabbits 187 and 184 had developed snuffly breathing, mucopurulent discharge from nostrils and mild anogenital swelling. 3/6 animals had a temperature of $>40^{\circ}\text{C}$ .
9	Rabbits 187, 184 and 154 had snuffly breathing, mucopurulent discharge and/or bleeding from nostrils, temperature of $>40^{\circ}\text{C}$ and mild anogenital swelling. All secondary lesions were black and had begun regressing. Two animals (176 and 179) recovered except for hard black scabs over inoculation site lesion and secondary lesions.
13	Lesions at the inoculation site covered with hard, black, concave scabs. Breathing of rabbits 187 and 154 were clear; anogenital swelling had regressed. These animals had lost some weight. Rabbit 184 still had slight mucopurulent discharge from nostrils and snuffly breathing, though much improved since day 9.
21	All animals recovered, although some appear 'ratty' with lumpy, misshapen ears, and numerous black scabs on body and face.





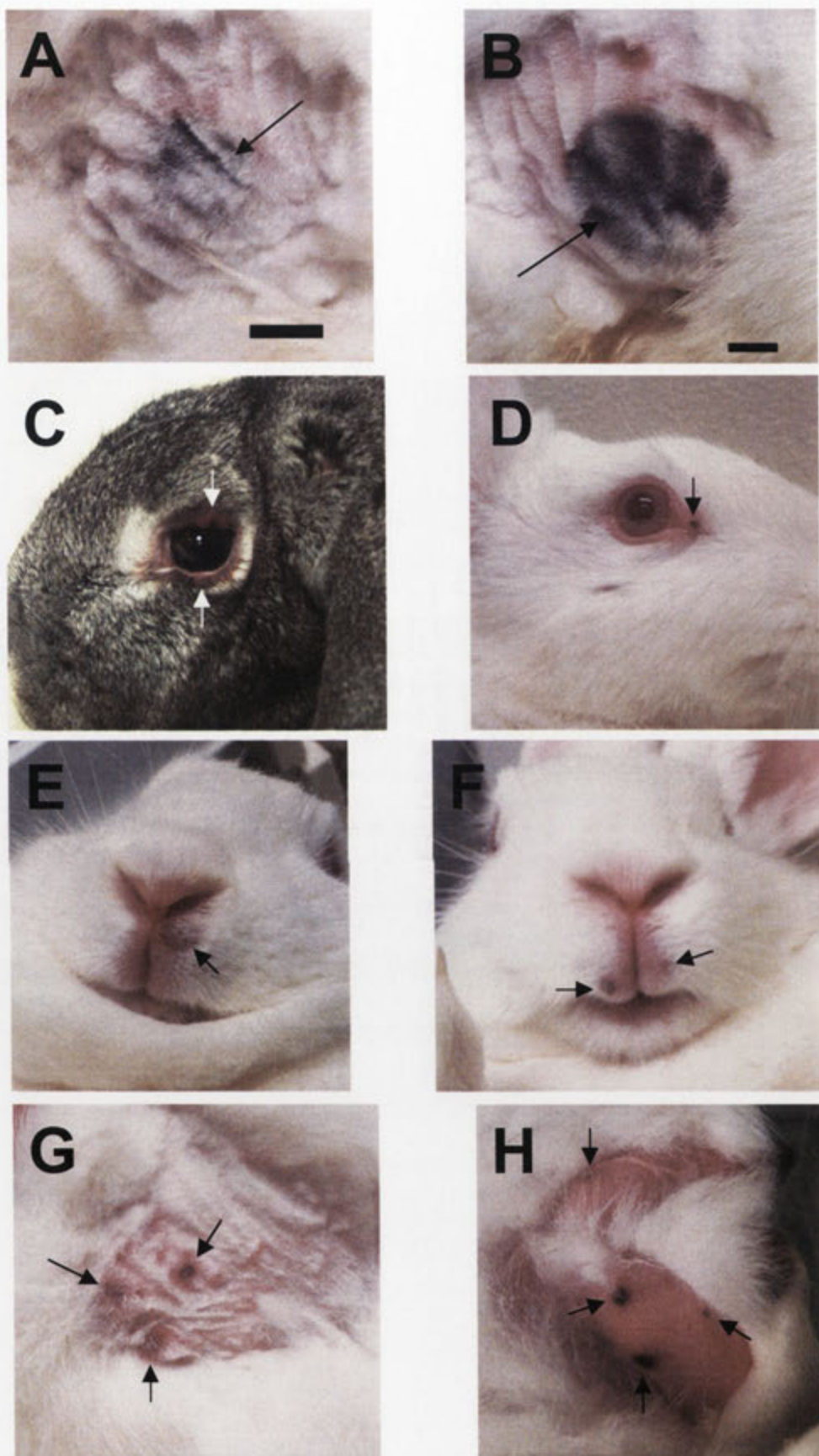
**Figure 7.9** - Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits, 3 days following challenge with 1000 pfu of SLS. Lesions at the inoculation site were between 15 and 25 mm in diameter, protuberant and pink or red in colour (A-E, arrowed). Lesions on some rabbits had developed a dark crimson or black colour in the centre (B and D, arrowed). Bars on images are 10 mm in size.

in colour (Figure 7.9D). At 5 dpc, all lesions at the inoculation site were black in colour or exhibited black patches, and most rabbits had developed small, red secondary lesions on the eyelids, ears or body. By 7 dpc, the lesions at the inoculation site ranged in size from 20 to 40 mm (Figure 7.10A and B). Secondary lesions had developed on all animals at sites including the eyelids, nose, mouth, body, anus or scrotum (Figure 7.10C-H). Most secondary lesions were small (no larger than 5 mm in diameter) and had progressed from crimson swellings to flat black lesions surrounded by crimson-coloured inflamed skin, within 24-48 hours of appearance (Figure 7.10D-H). Secondary lesions were evident on the bodies of three animals, and these lesions were generally larger than at other sites, usually 10 mm in diameter (Figure 7.10G).

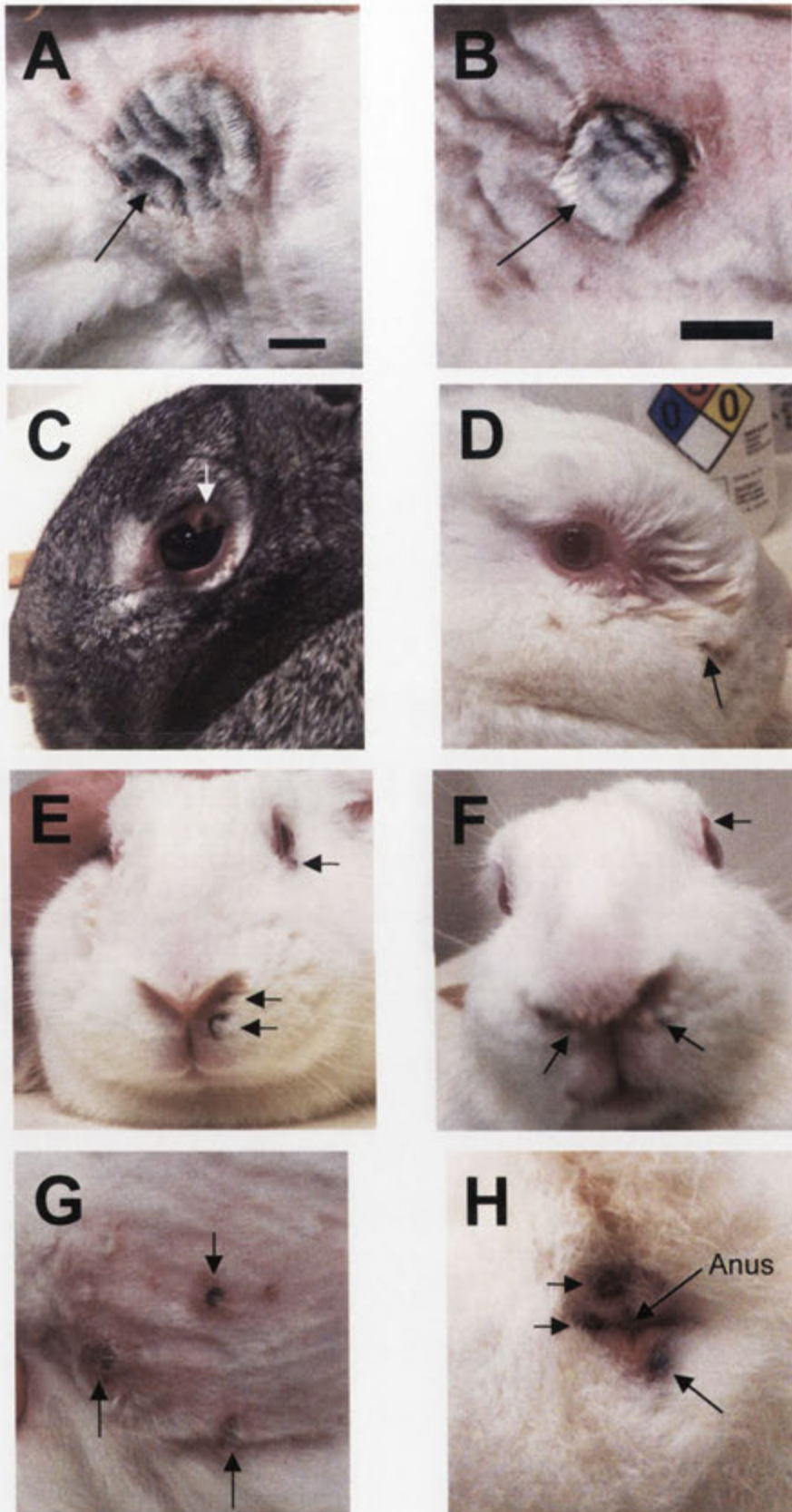
By 8 dpc, two rabbits (187 and 184) had developed slightly snuffly breathing associated with nasal discharge and bleeding from lesions in the nostrils, and a third rabbit (154) developed snuffly breathing at 9 dpc. These three animals also exhibited mild anogenital swelling. Two animals (176 and 179) had shown improvement and by 10 dpc had recovered completely except for residual scabs at the sites of small secondary lesions and the lesion at the inoculation site.

By 13 dpc, most rabbits had recovered from challenge, however scabby black lesions were evident at many sites of the body although the surrounding skin was pink (Figure 7.11), and rabbits which had exhibited mucopurulent discharge from the nose still had crusty material surrounding the nostrils (Figure 7.11F). Some animals also had a slight watery discharge from the eyes (Figure 7.11D). One rabbit (187) still had slightly snuffly breathing and two had lost some weight over the preceding 5 days (187, 154). Other symptoms of disease such as anogenital swelling had regressed completely.





**Figure 7.10** - Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits, 7 days after challenge with 1000 pfu of SLS. Representative primary lesions (A and B, arrowed) were protuberant, black and haemorrhagic. Crimson or black secondary lesions on the eyelids (C and D, arrowed), mouth and nose (E and F, arrowed), body (G, arrowed), and scrotum (H, arrowed). Bars on images are 10 mm in size.



**Figure 7.11** - Uriarra  $\Delta M063Rgptgus$ -vaccinated rabbits, 13 days after challenge with 1000 pfu of SLS. Representative primary lesions (A and B, arrowed) were covered with black scabs with pink skin at the edges. Secondary lesions covered with black scabs on the eyes (C, E, F, arrowed), nose (E, F, arrowed), face (D, arrowed), body (G, arrowed) and anus (H, arrowed). Bars on images are 10 mm in size.

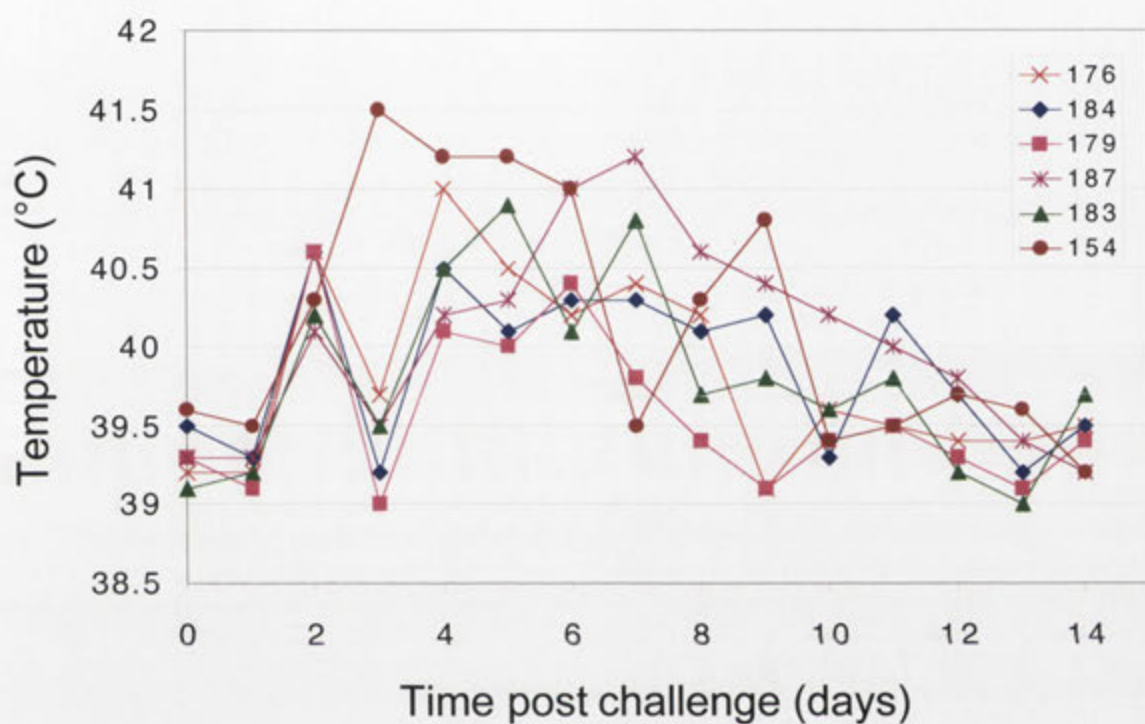
All rabbits had rectal temperatures over 40°C at 2 dpc, with all except 154 falling below 40°C at 3 dpc before increasing again at 4 dpc (Figure 7.12). Over the course of infection, rabbits had a temperature of 40°C or greater on at least 4 days. Animals that showed the most severe disease symptoms (187, 184, 154) had fevers for longer periods, with rabbit 187 having a fever for 8 days from 4-11 dpc. By 12 dpi, the temperatures of all animals had fallen below 40°C.

In summary, the degree of secondary signs of myxomatosis following challenge varied from a few very small secondary lesions on the ears of one rabbit (179), more numerous secondary lesions (176, 183), to more severe signs of myxomatosis including mucopurulent discharge from the nasal passages, mild obstruction of the respiratory tract resulting in snuffly breathing, and mild anogenital swelling (154, 184 and 187). However, by 2 weeks after lethal challenge, even the rabbits with moderate symptoms of myxomatosis had improved, and by 21 dpi had completely recovered from SLS infection. All rabbits survived challenge, and the symptoms exhibited were very mild in comparison to SLS challenge of non-immune rabbits such as those vaccinated with pDual2+ (Section 3.2.7.1).

#### *7.2.4.3 Analysis of anti-myxoma virus antibody development in Uriarra $\Delta M063R$ gptgus-vaccinated rabbits*

The anti-myxoma virus ELISA antibody titre was determined for all serum samples after vaccination and challenge and are shown in Figure 7.13. Anti-myxoma virus antibody capable of neutralising myxoma virus *in vitro* was analysed using a plaque reduction neutralisation assay (PRNA) for serum samples taken before challenge (6 weeks after vaccination) and 2 weeks after challenge (8 weeks after vaccination). The percentage





**Figure 7.12** - Rectal temperatures of rabbits vaccinated with Uriarra  $\Delta$ M063Rgptgus following challenge with SLS.

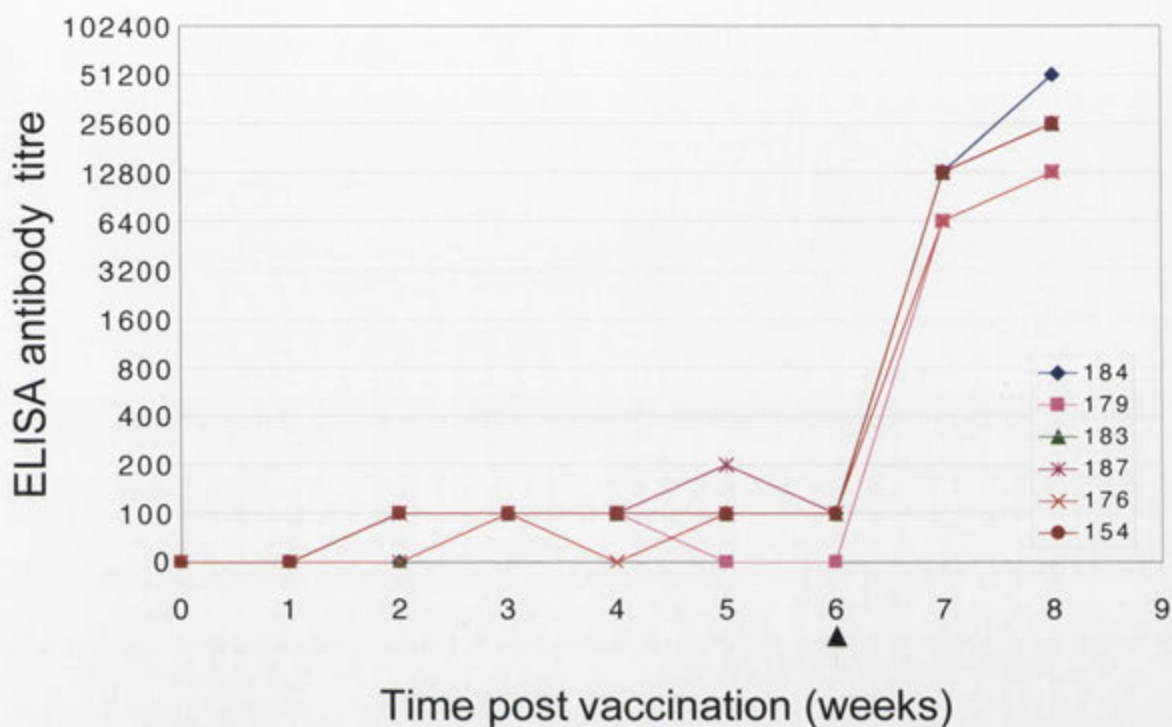
plaque neutralisation for each serum dilution tested and the corresponding 50% and 80% plaque neutralisation titres are shown for each rabbit in Figure 7.14.

Following inoculation with 100,000 pfu of Uriarra  $\Delta$ M063Rgptgus, all rabbits had detectable antibody, with titres of 100 or 200 measured after 2 weeks post vaccination (wpv) (Figure 7.13), although titres of some rabbits returned to zero for some time before challenge. Very low levels of neutralising antibody were recorded at 6 wpv, with neutralisation of only 0-35% of virus in serum diluted 1/10 (Figure 7.14A).

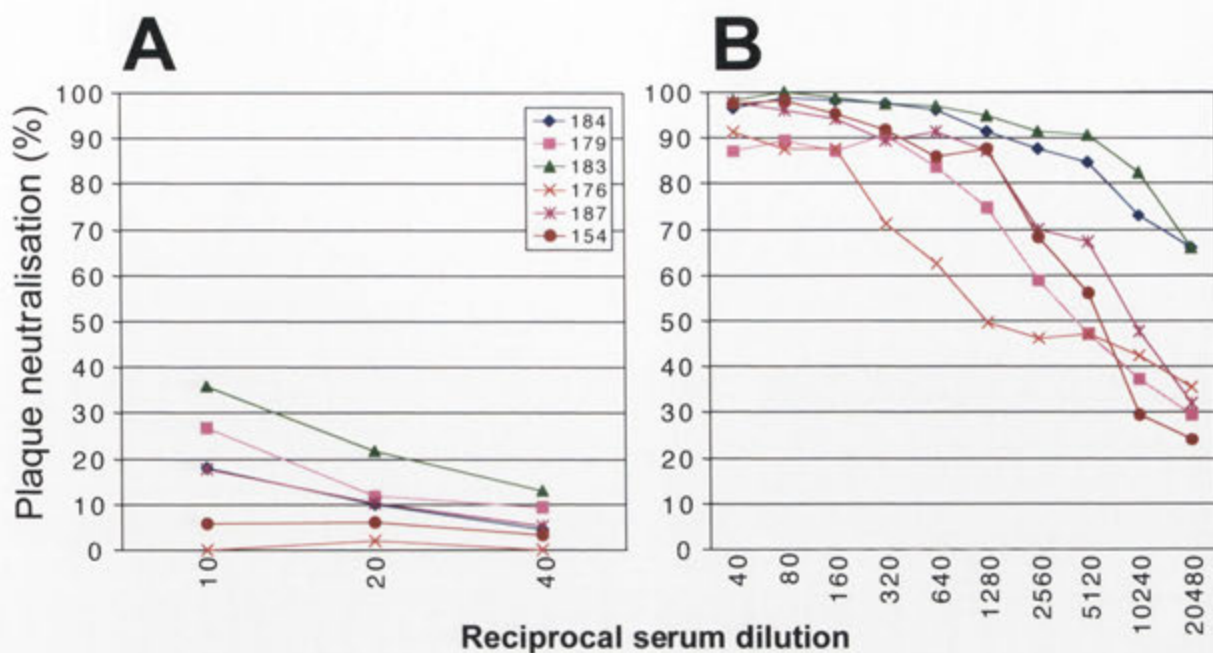
After challenge, rabbits had very large and rapid increases in ELISA titres with 32- to 64-fold increases observed within 7 days of challenge, and 128- to 512-fold increases to between 12800 and 51200 over 14 days (Figure 7.14). Similarly, PRNA titres of all rabbits measured 2 weeks after SLS challenge had increased significantly, with 50% neutralisation titres ranging from 640 to >20480, and 80% neutralisation titres between 160 and 10240 (Figure 7.14B and C).

### **7.2.5 Use of Uriarra $\Delta$ M063Rgptgus in a boosting regimen**

Uriarra  $\Delta$ M063Rgptgus provided complete protection against lethal disease following SLS challenge six weeks after vaccination, although half of the challenged rabbits exhibited mild to moderate symptoms of myxomatosis. Therefore, the use of this replication-deficient vaccine was also evaluated as part of a boosting regimen by boosting Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits with another dose of Uriarra  $\Delta$ M063Rgptgus, to determine if this was able to elicit greater levels of protective immunity in domestic rabbits. Boosting of rabbits with the TKO vaccine will be evaluated in Section 7.2.6, though the



**Figure 7.13 - ELISA anti-myxoma virus antibody titres in rabbits vaccinated with Uriarra  $\Delta$ M063Rgptgus.** The ELISA titre was determined by titration in duplicate of two fold serial dilutions of rabbit sera from 1:100. The ELISA endpoint was defined as the reciprocal serum dilution 0.1 OD<sub>405</sub> units above the OD<sub>405</sub> of the 1:100 negative control. An OD<sub>405</sub> of less than 0.1 for a 1:100 dilution of serum was recorded as a titre of zero. Rabbits were challenged with SLS following sera collection at week 6 (arrow).



**C**

Rabbit	Neutralising antibody titre			
	Pre Challenge (Week 6)		Post Challenge (Week 8)	
	50%	80%	50%	80%
184	< 10	< 10	> 20480	5120
179	< 10	< 10	2560	640
183	< 10	< 10	> 20480	10240
176	< 10	< 10	640	160
187	< 10	< 10	5120	1280
154	< 10	< 10	5120	1280

**Figure 7.14 - Analysis of neutralising anti-myxoma virus antibodies in the serum of rabbits vaccinated with Uriarra  $\Delta$ M063Rgptgus.** Neutralising antibody was measured by plaque reduction neutralisation assay and the percentage plaque neutralisation recorded at week 6 (A) and week 8 (B) for each serum dilution (two-fold from 1:10 to 1:20480). The highest reciprocal serum dilution at which 50% or greater or 80% or greater of myxoma virus plaque formation was neutralised is summarised (C).



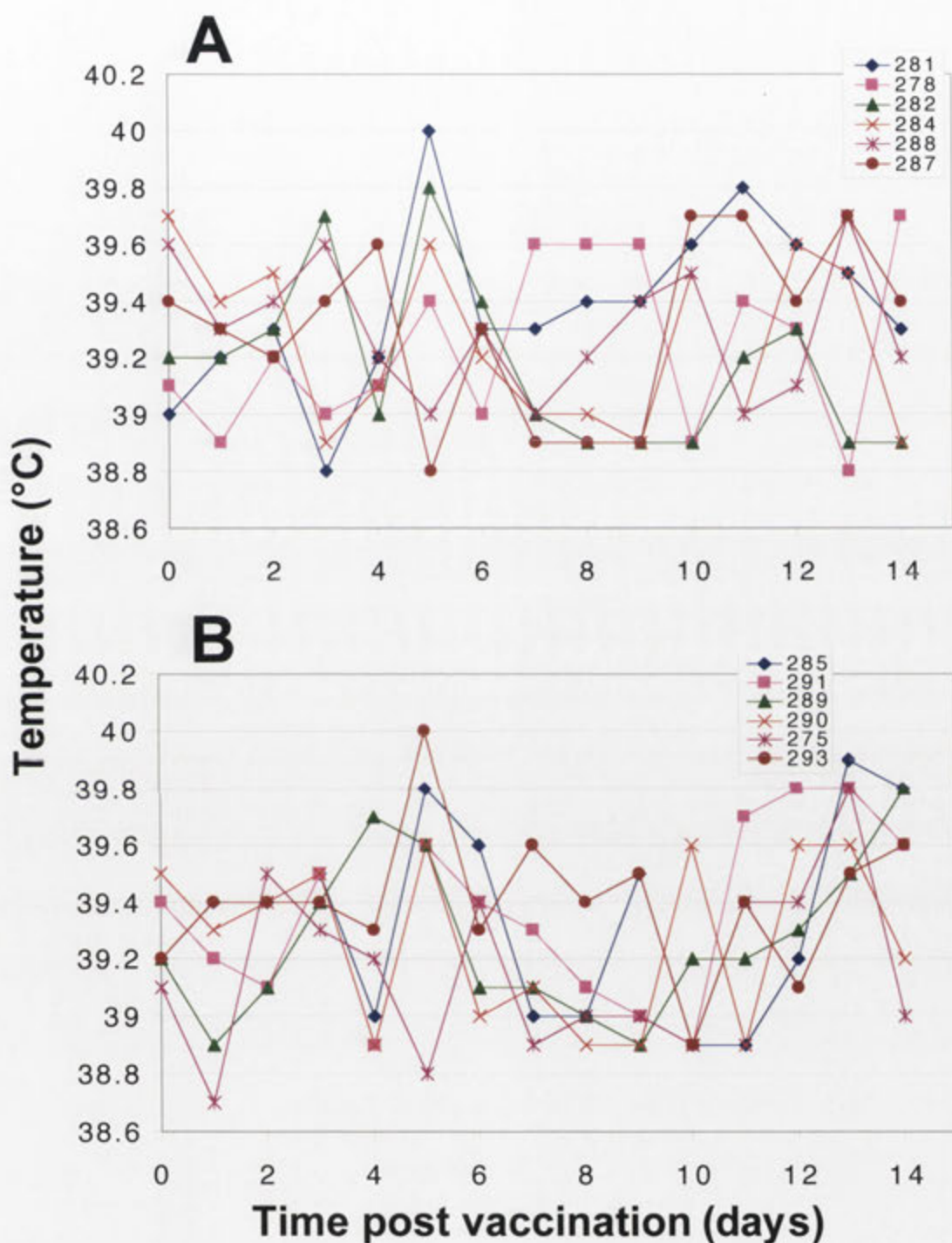
initial vaccination of these rabbits with Uriarra  $\Delta M063Rgptgus$  has been described in Section 7.2.5.1.

#### 7.2.5.1 Vaccination with Uriarra $\Delta M063Rgptgus$

Twelve 12 week old domestic rabbits were inoculated with 100,000 pfu of Uriarra  $\Delta M063Rgptgus$  intradermally on the left thigh and monitored daily for 14 days. The clinical signs exhibited by these twelve rabbits following inoculation were very similar to those of mature rabbits vaccinated with Uriarra  $\Delta M063Rgptgus$  (Table 7.1; Figures 7.5-7.7). No reaction beyond a slight pinkness at the skin of the inoculation site was observed for any rabbit before 5 dpi. At 6 dpi, all rabbits had a slightly raised, pink lump at the inoculation site which, over the next several days, grew to a slightly protuberant, 20 mm red lesion. By 10 dpi, most lesions were dark red in colour and two developed small black scabs at the centre. The primary lesions regressed, and by 17 dpi, most had regressed completely or a small pink lump remained at the inoculation site. No secondary lesions were seen on any rabbit following vaccination with Uriarra  $\Delta M063Rgptgus$ .

Rectal temperatures of rabbits remained within normal limits following inoculation of Uriarra  $\Delta M063Rgptgus$ , except for two rabbits (281 and 293) which had rectal temperatures of 40°C for one day only (Figure 7.15).

Six of these rabbits were later boosted with Uriarra  $\Delta M063Rgptgus$  (Section 7.2.5.2), and the other six boosted with TKO (Section 7.2.6).



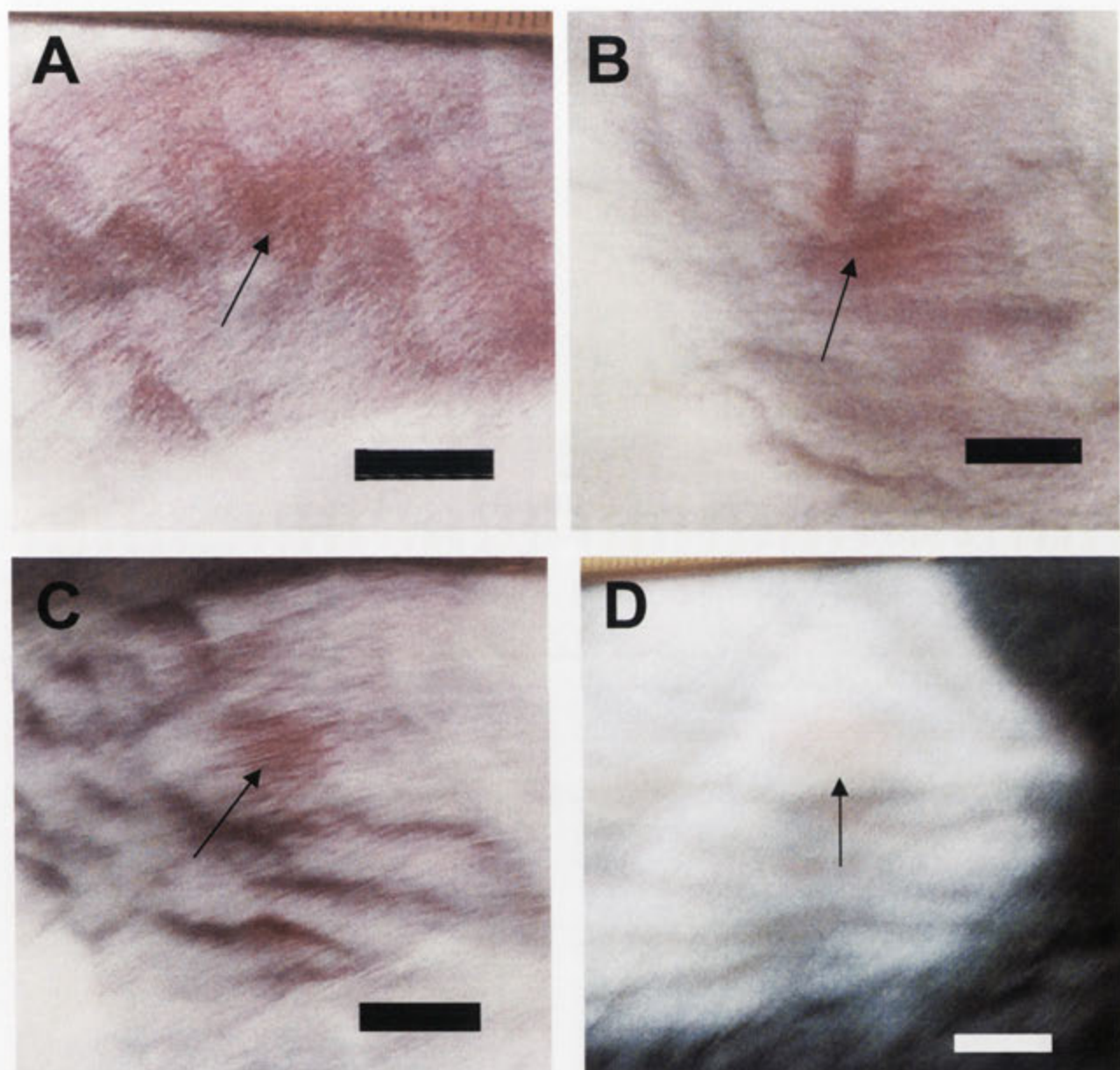
**Figure 7.15 - Rectal temperatures of 12 week old rabbits following vaccination with 100,000 pfu of Uriarra  $\Delta$ M063Rgptgus.** These animals were divided into two groups based on the virus boost to be used four weeks after inoculation with Uriarra  $\Delta$ M063Rgptgus. Panel A shows the six rabbits to be boosted with Uriarra  $\Delta$ M063Rgptgus and Panel B shows the six rabbits to be boosted with TKO.

#### 7.2.5.2 *Boosting rabbits with Uriarra ΔM063Rgptgus*

Four weeks after inoculation with Uriarra ΔM063Rgptgus, six of the twelve rabbits were boosted with 100,000 pfu of Uriarra ΔM063Rgptgus intradermally on the right thigh and monitored daily for 10 days. The symptoms exhibited upon boosting were very similar to those after the first dose of Uriarra ΔM063Rgptgus, except for a rapid response at the inoculation site, where within 24 hours, a 6-12 mm pink lump had developed on all rabbits. By 3 days post boosting (dpb), this had grown in size to an 8-15 mm raised pink lesion (Figure 7.16), which changed in colour to red on some animals by 6 dpb (Figure 7.17). By 9 dpb, the lesion had completely regressed on all rabbits, with only a small residual scab remaining on two animals. The rectal temperatures of Uriarra ΔM063Rgptgus-boosted rabbits remained below 40°C following the boost (Figure 7.18).

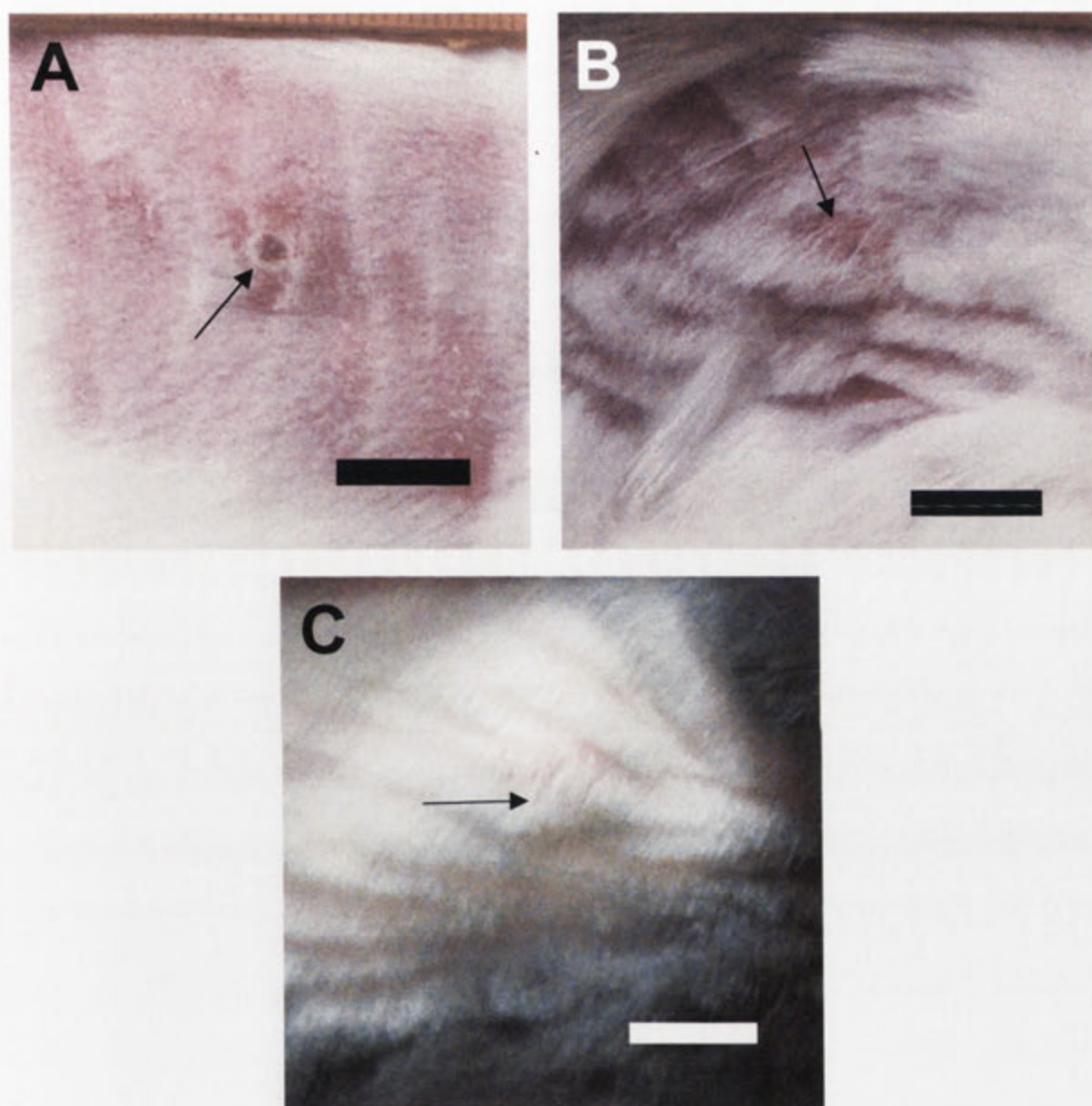
#### 7.2.5.3 *Challenge of Uriarra ΔM063Rgptgus- boosted rabbits with SLS*

The six Uriarra ΔM063Rgptgus-boosted rabbits were challenged with 1000 pfu of SLS at a site on the back above the left thigh 6 weeks after boosting, and examined daily for 14 days following challenge. The clinical signs of these animals are summarised in Table 7.3. Within 24 hours, all rabbits had developed a raised red lesion 10 mm in diameter at the inoculation site. At 2 dpc, the lesion had developed a black, haemorrhagic centre on two rabbits (281 and 286). By 3 dpc, the lesions were 15-30 mm in diameter and had developed black centres on all rabbits (Figure 7.19). Small secondary lesions (<5 mm) distal from the skin surrounding the inoculation site were evident at 5 dpc on only two of the six challenged rabbits (282 and 288), at the edges of the ears and face. Only five small secondary lesions were noted on each of these animals during the course of infection. The secondary lesion on the ear of one rabbit at 6 dpc is shown in Figure 7.20E.

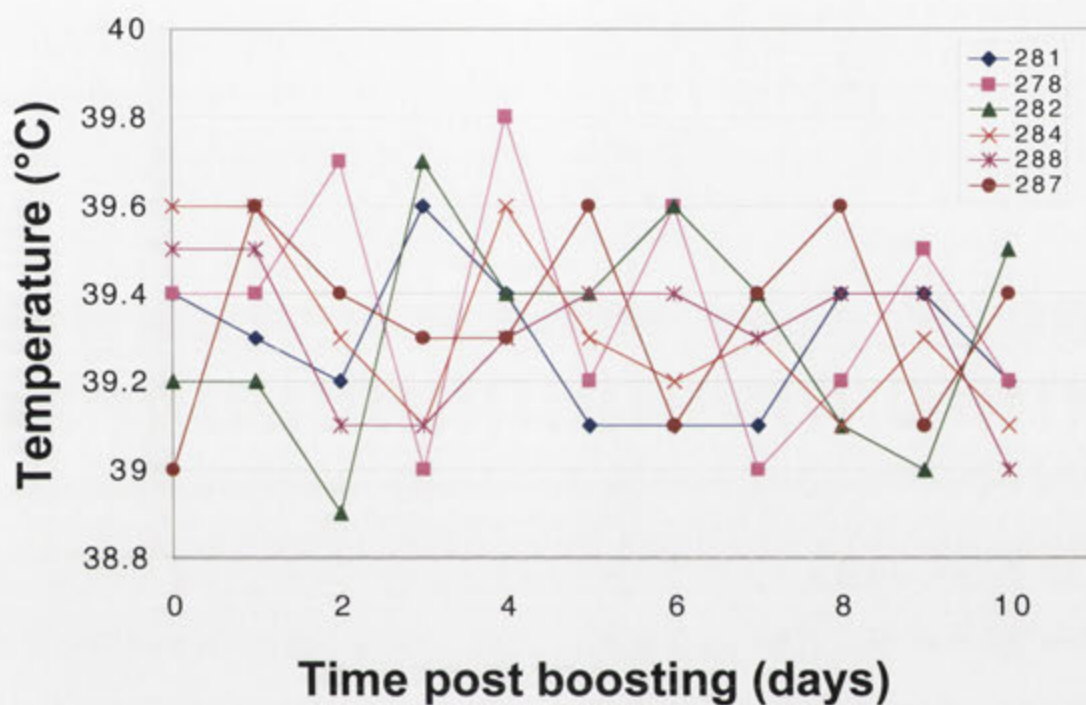


**Figure 7.16** - Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits boosted with 100,000 pfu of Uriarra  $\Delta$ M063Rgptgus, 3 days after inoculation. Lesions at the inoculation site were 8-15 mm in diameter and pink or red in colour as shown (A-D, arrowed). Bars on images are 10 mm in size.





**Figure 7.17** - Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits boosted with 100,000 pfu of Uriarra  $\Delta$ M063Rgptgus, 6 days after boosting. The lesion on one rabbit had scabbed over and regressed (A, arrowed). Other lesions were 10 mm in diameter and pink or red in colour as shown (B and C, arrowed). Bars on images are 10 mm in size.

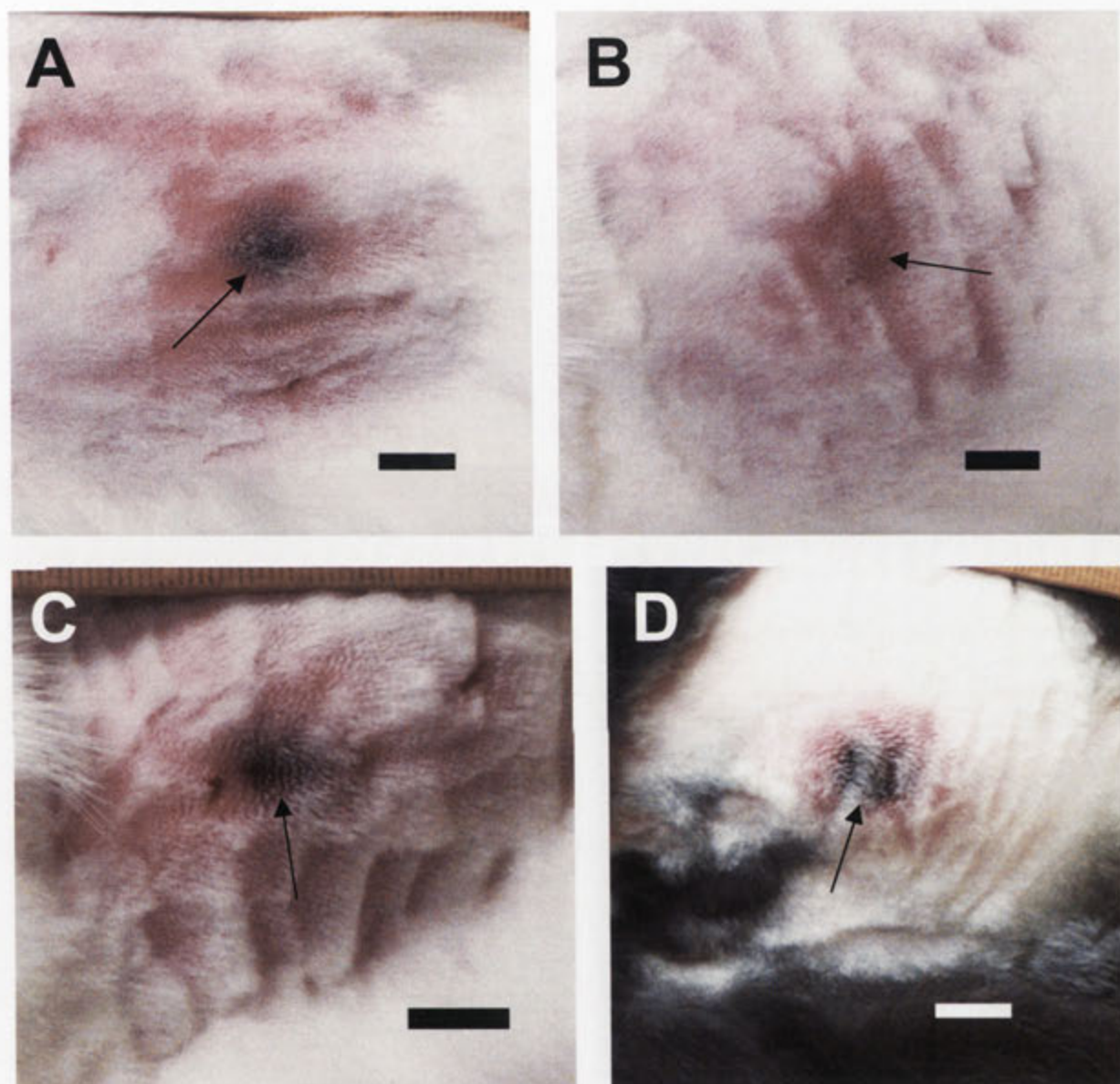


**Figure 7.18** - Rectal temperatures of Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits following boosting with 100,000 pfu of Uriarra  $\Delta$ M063Rgptgus.

**Table 7.3** – Clinical signs of Uriarra ΔM063R*gptgus*-boosted rabbits following challenge with SLS.

Day post inoculation	Clinical signs
1	Pink lesion at the inoculation site, 10 mm diameter
3	Lesion at the inoculation site 15-30 mm diameter with black, haemorrhagic centres
5	Lesion at the inoculation site 15-35 mm diameter circumscribed and black. Secondary lesions on ears or face of rabbits 282 and 288.
10	Lesion at the inoculation site covered with a hard, black, concave scab. Secondary lesions regressing covered with black scab.



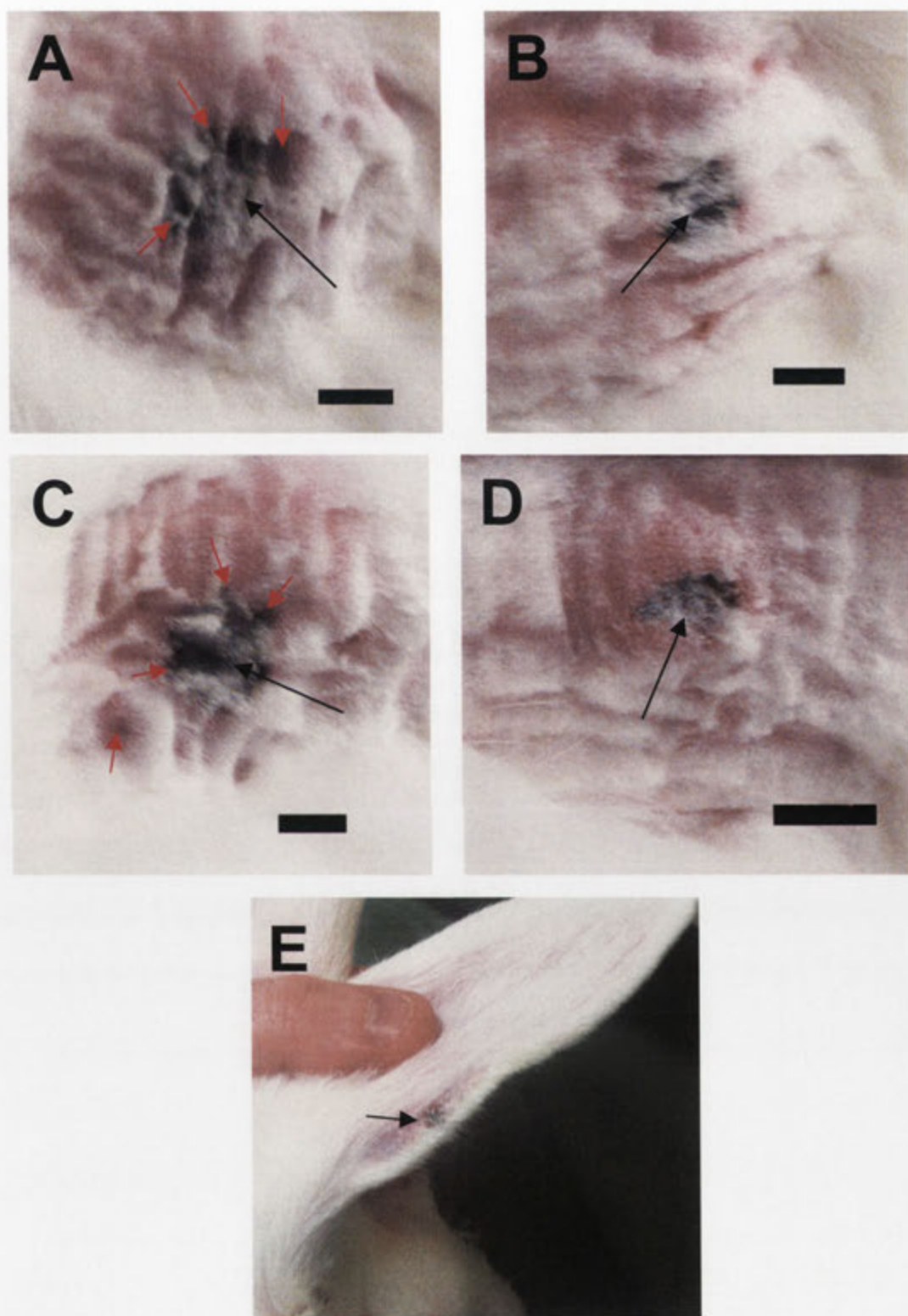


**Figure 7.19** - Rabbits boosted with Uriarra  $\Delta$ M063Rgptgus, 3 days after challenge with 1000 pfu of SLS. Lesions at the inoculation site were 15-30 mm in diameter, protuberant with haemorrhagic or black centres as shown (A-D, arrowed). Bars on images are 10 mm in size.

By 6 dpc, the primary lesions on all animals were 15-35 mm in diameter and covered with a hard black scab (Figure 7.20A-D). Some lesions were not circumscribed but were irregular around the edges with what appeared to be smaller lesions (approximately 5-10 mm) developing at the perimeter of the primary lesion (Figure 7.20A and C). Two rabbits had also developed 10 mm crimson or purple lesions on the skin surrounding the primary lesion, and rabbit 281 had developed a large (30x20 mm) purple, bruise-like lesion 10 cm above the primary. Secondary lesions on the ears and face were covered with black scabby material, although the surrounding skin was swollen and coloured red or crimson (Figure 7.20E).

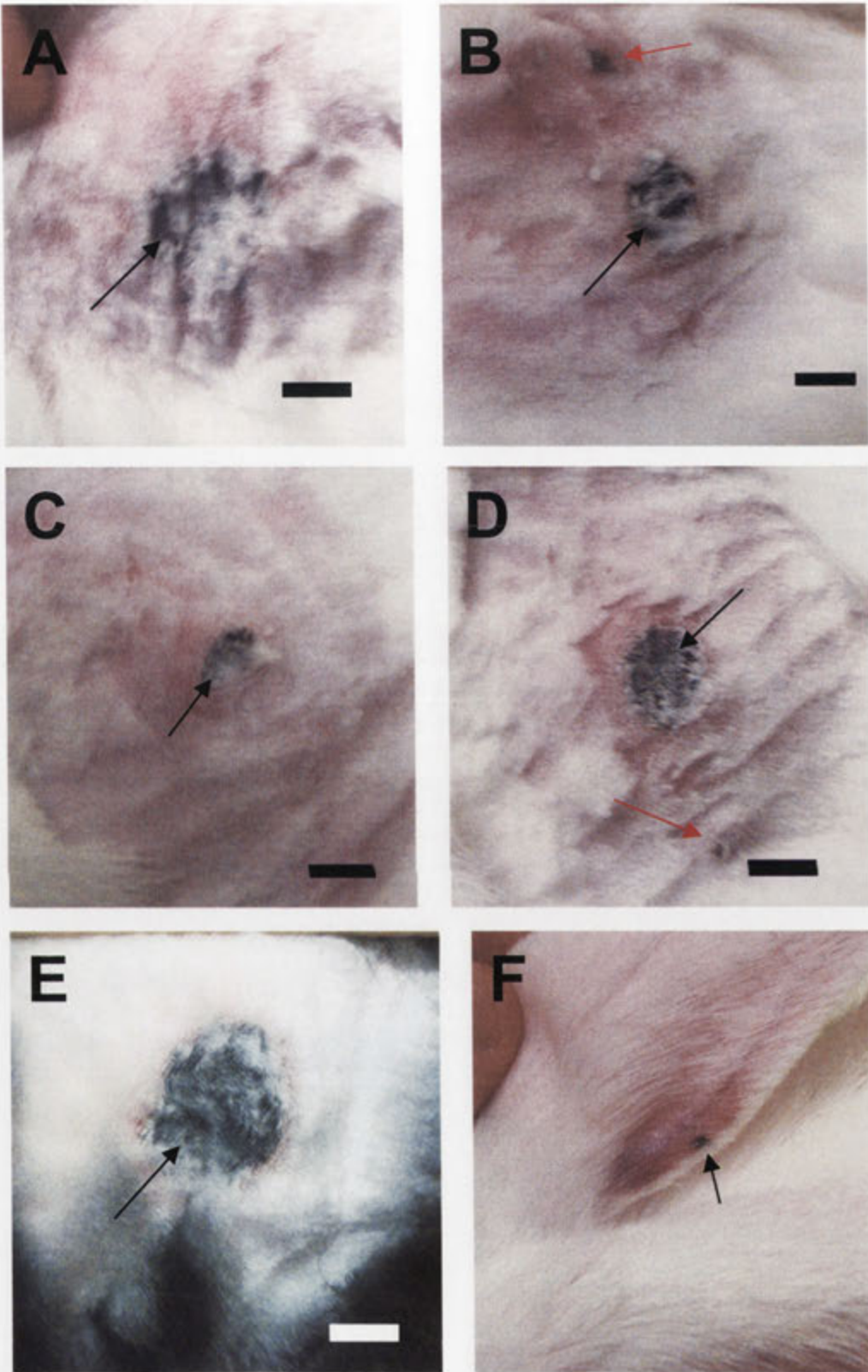
By 9 dpc, animals had recovered completely except for residual black scabs at sites of lesions. The skin colour immediately surrounding the lesion at the inoculation site had faded to a pink colour (Figure 7.21). Secondary lesions surrounding the primary lesion and at distal sites had also regressed except for black scabby material at the surface (Figure 7.21B, D and F). The appearance of rabbits did not change significantly before the end of the experiment at 15 dpc (Figure 7.22). None of the Uriarra  $\Delta M063Rgptgus$ -boosted rabbits developed any serious symptoms of myxomatosis such as mucopurulent discharge from the eyes or nose, laboured breathing or anogenital swelling following SLS challenge.

The rectal temperature of each rabbit was recorded daily following SLS challenge and are shown in Figure 7.23. Only two of the six rabbits had temperatures of 40°C or above following challenge including 278 which had a temperature of 41.1°C at 3 dpc only, and rabbit 288 which had temperatures of 40°C and above at 1, 2, 4 and 5 dpc.

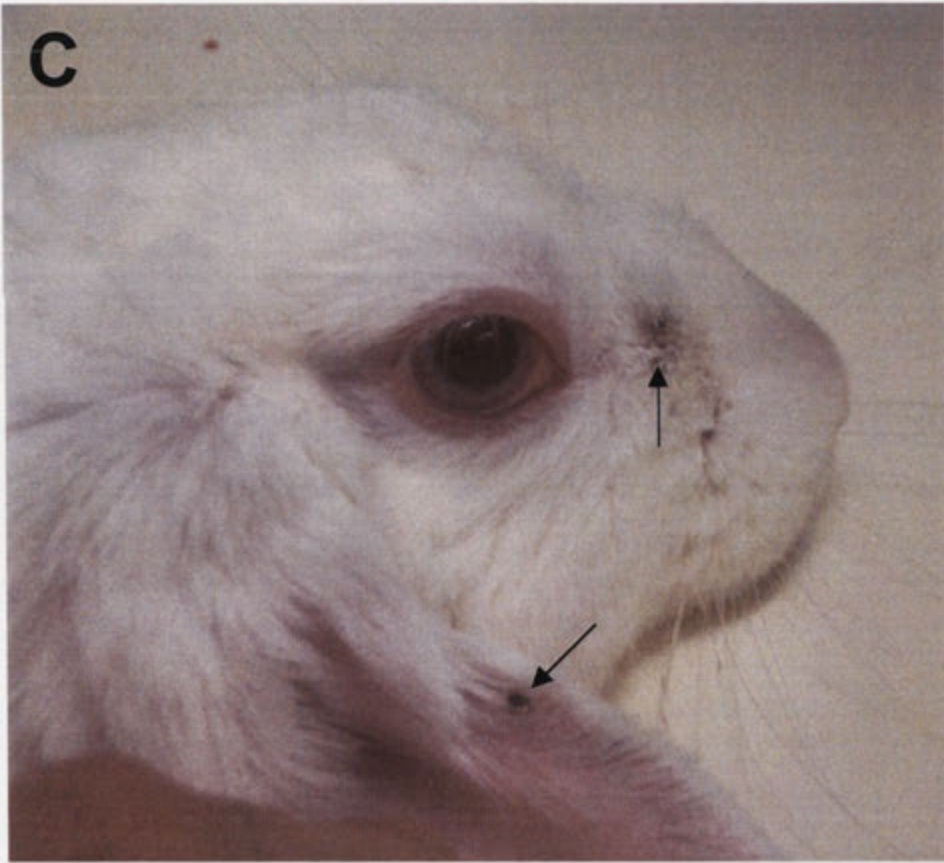
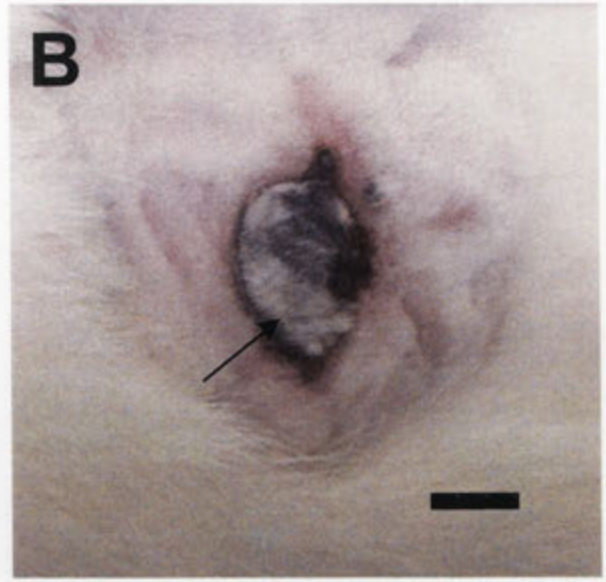
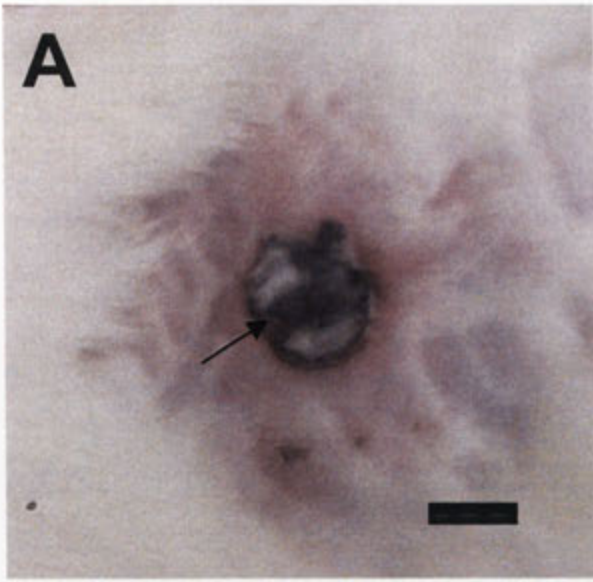


**Figure 7.20** - Rabbits boosted with Uriarra  $\Delta$ M063Rgptgus, 6 days after challenge with 1000 pfu of SLS. Black scabbed-over lesions at the inoculation site (A-D, black arrows). Some lesions had smaller lesions developing at the perimeter (A and C, red arrows). A small secondary lesion on the ear that was covered with a black scab (E, arrowed). Bars on images are 10 mm in size.

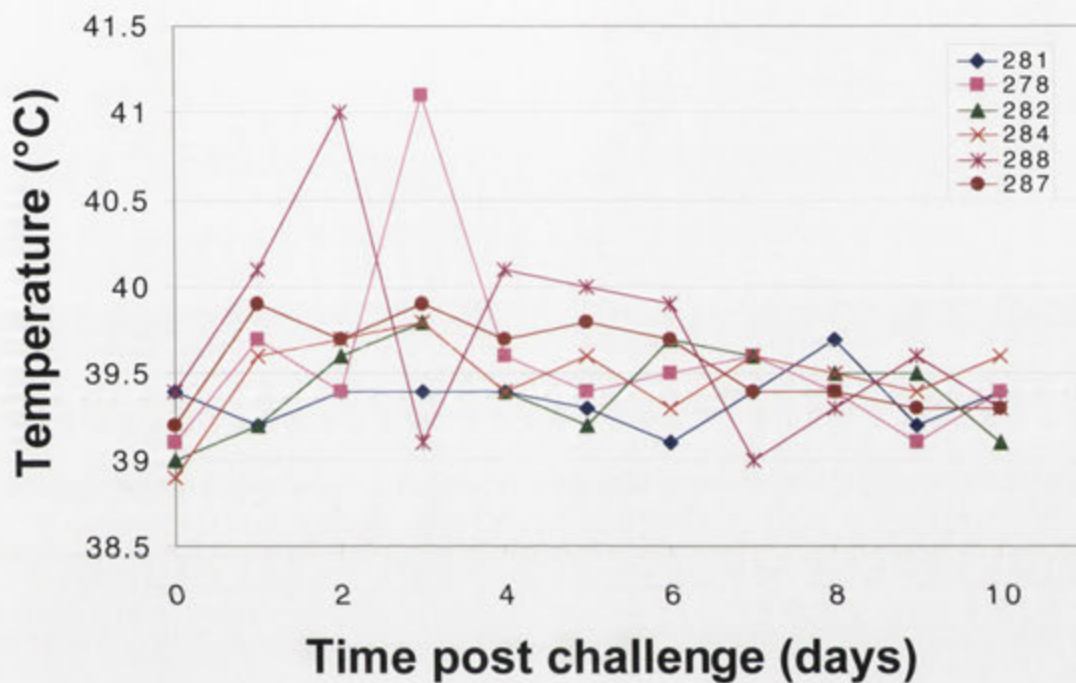




**Figure 7.21** - Rabbits boosted with *Uriarra ΔM063Rgptgus*, 9 days after challenge with 1000 pfu of SLS. Lesions at the inoculation site were covered with a hard, black, concave scab (A-E, black arrows) Small, black secondary lesions on the skin near the primary lesion (B and D, red arrows) or the ears (E, arrowed). Bars on images are 10 mm in size.



**Figure 7.22** - Rabbits boosted with Uriarra  $\Delta$ M063Rgptgus, 15 days after challenge with 1000 pfu of SLS. Lesions at the inoculation site covered by hard black scabs (A and B, arrowed). Secondary lesions covered with black scabs (C, arrowed). Bars on images are 10 mm in size.



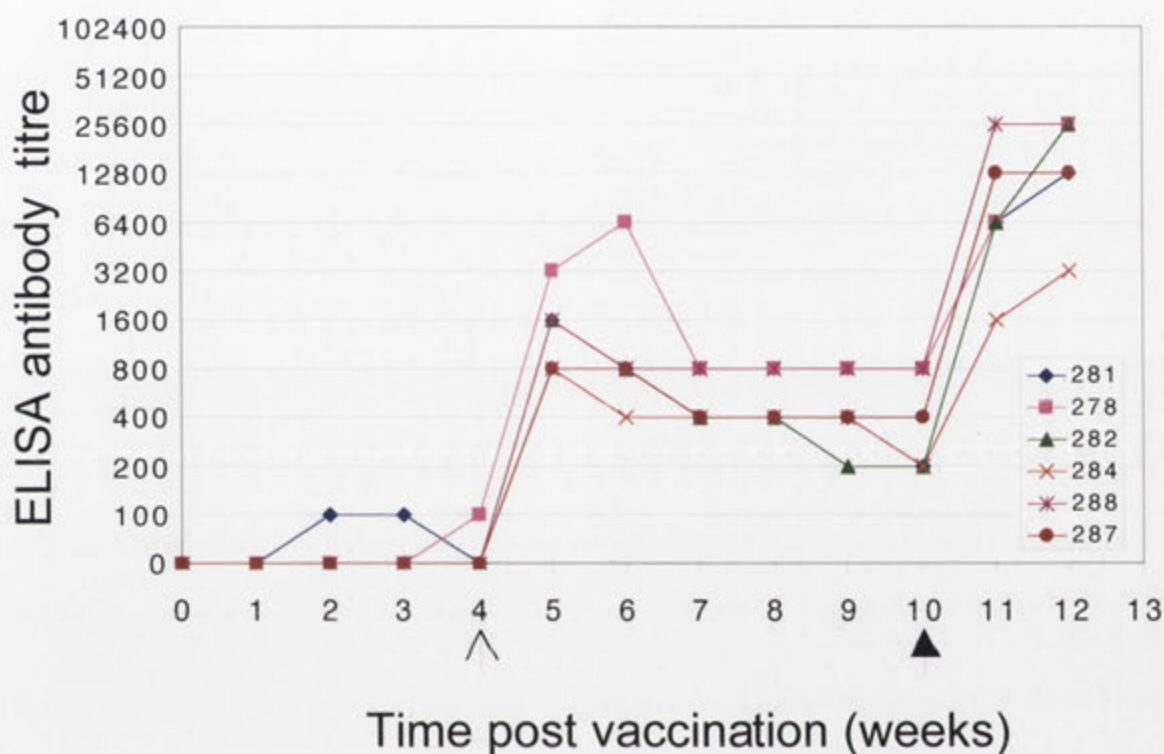
**Figure 7.23** - Rectal temperatures of Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits boosted with Uriarra  $\Delta$ M063Rgptgus, following challenge with 1000 pfu of SLS.

#### 7.2.5.4 Analysis of anti-myxoma virus antibody development in Uriarra $\Delta M063Rgptgus$ -boosted rabbits

The anti-myxoma virus ELISA antibody titre was determined for all serum samples after vaccination, boosting and challenge, and are shown in Figure 7.24. Antibody capable of neutralising myxoma virus *in vitro* was analysed using PRNA for serum samples taken before the Uriarra  $\Delta M063Rgptgus$  boost (4 wpv), 2 weeks after boosting (6 wpv), before challenge (10 wpv) and 2 weeks after challenge (12 wpv). The percentage plaque neutralisation for each serum dilution tested and the corresponding 50% and 80% plaque neutralisation titres are shown for each rabbit in Figure 7.25.

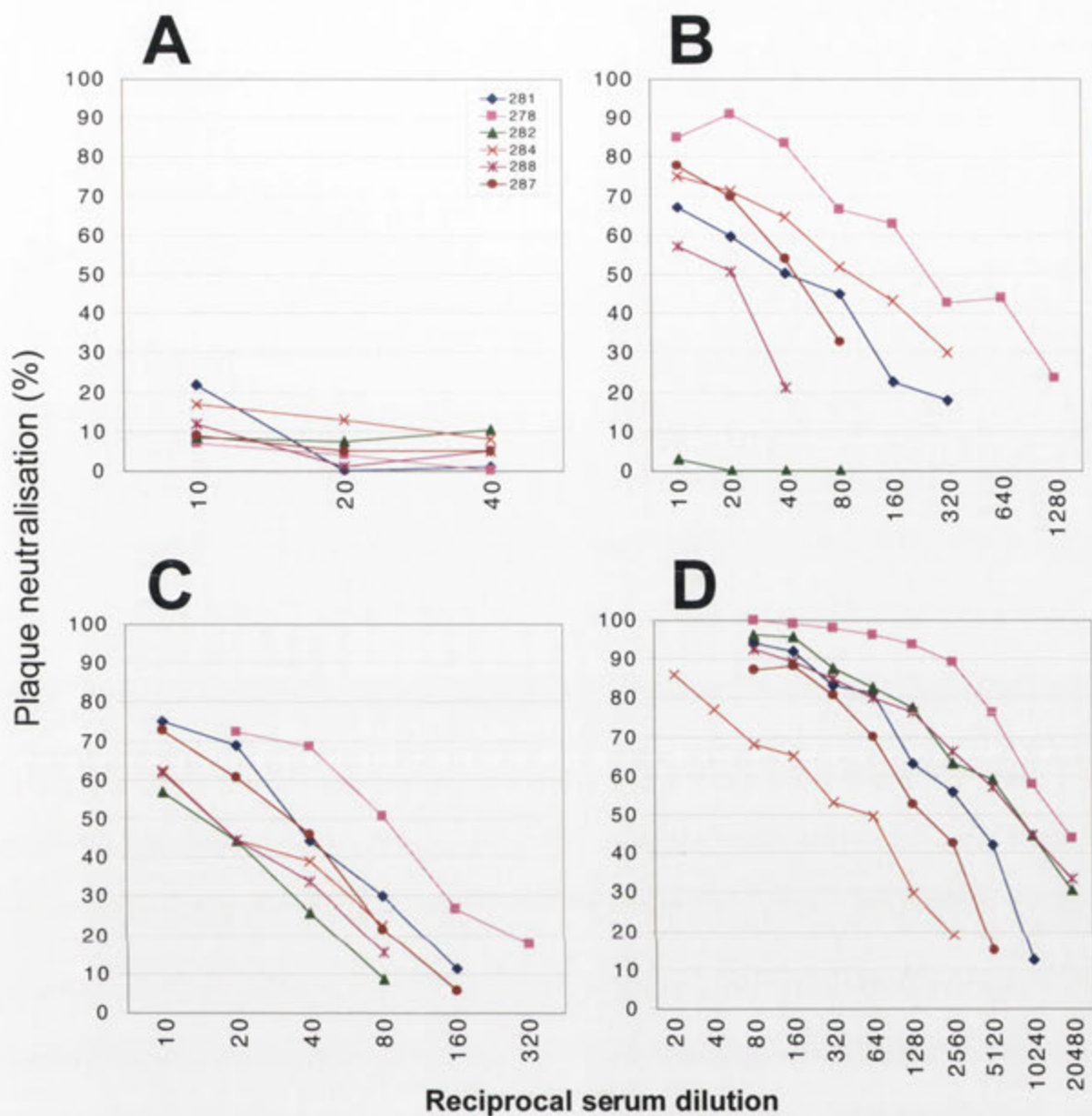
Only two rabbits out of six had anti-myxoma virus antibody detectable by ELISA by 4 wpv (Figure 7.24), and there were only very low levels of virus-neutralising antibody as detected by PRNA (Figure 7.25A). In contrast, all mature rabbits vaccinated with Uriarra  $\Delta M063Rgptgus$  had anti-myxoma virus antibody as detected by ELISA, for at least one week before challenge (Figure 7.13). After boosting with Uriarra  $\Delta M063Rgptgus$ , all six rabbits showed significant increases (4-fold or greater) in ELISA antibody titre with 16- to 32-fold increases within 1 week of boosting, with titres ranging between 800 and 3200, and rabbit 278 increased to 6400 at 6 wpv (Figure 7.24). After boosting, titres in some rabbits (278 and 282) dropped slightly, and by the time of challenge, titres ranged between 200 and 800. Similar increases in PRNA titres following boosting were recorded for all rabbits except rabbit 282 which still had very low virus-neutralisation activity (Figure 7.25B), although this increased by 10 wpv, with virus neutralisation in 1/10 diluted serum increasing from 3% to 57% (Figure 7.25B and C). The 50% PRNA titres of other rabbits at 2 weeks after boosting ranged between 10 and 160, with no correlation with the recorded ELISA titres (Figure 7.25E). PRNA titres fell 2- to 4-fold by 10 wpv (Figure 7.25C).





**Figure 7.24** - ELISA anti-myxoma virus antibody titres in rabbits vaccinated with Uriarra  $\Delta$ M063Rgptgus and boosted with Uriarra  $\Delta$ M063Rgptgus. The ELISA titre was determined by titration in duplicate of two fold serial dilutions of rabbit sera from 1:100. The ELISA endpoint was defined as the reciprocal serum dilution 0.1 OD<sub>405</sub> units above the OD<sub>405</sub> of the 1:100 negative control. An OD<sub>405</sub> of less than 0.1 for a 1:100 dilution of serum was recorded as a titre of zero. At week 4, rabbits were boosted with 10<sup>5</sup> pfu of Uriarra  $\Delta$ M063Rgptgus (open arrow) and challenged with SLS at week 10 (closed arrow).

**Figure 7.25 - Analysis of neutralising anti-myxoma virus antibodies in the serum of rabbits vaccinated with Uriarra  $\Delta$ M063Rgptgus and boosted with Uriarra  $\Delta$ M063Rgptgus.** Neutralising antibody was measured by plaque reduction neutralisation assay and the percentage plaque neutralisation recorded before boosting (week 4, A), after boosting (week 6, B), before challenge (week 10, C) and after challenge (week 12, D) for each serum dilution (two-fold from 1:10 to 1:20480). The highest reciprocal serum dilutions at which 50% or greater and 80% or greater of myxoma virus plaque formation was neutralised is summarised (E).



<b>E</b>	Neutralising antibody titre							
	Pre Boost (Week 4)		Post Boost (Week 6)		Pre Challenge (Week 10)		Post Challenge (Week 12)	
	50%	80%	50%	80%	50%	80%	50%	80%
Rabbit	50%	80%	50%	80%	50%	80%	50%	80%
281	< 10	< 10	40	< 10	20	< 10	2560	320
278	< 10	< 10	160	40	80	< 10	10240	2560
282	< 10	< 10	< 10	< 10	10	< 10	5120	640
284	< 10	< 10	80	< 10	10	< 10	320	20
288	< 10	< 10	10	< 10	10	< 10	5120	640
287	< 10	< 10	40	< 10	20	< 10	1280	320

The ELISA and PRNA titres of all Uriarra  $\Delta$ M063Rgptgus-boosted rabbits increased significantly following challenge with SLS, with 8- to 32-fold increases in ELISA titre within 1 week (Figure 7.24), and 32- to 512-fold increases in 50% PRNA titres by 2 weeks after challenge (Figure 5.25D and E). At 12 wpv, 1 rabbit (284) had a final anti-myxoma virus antibody titre of 3200, and the remaining rabbits had a titre of 12800 or 25600. At this time, the 50% PRNA titre of rabbit 284 was only 320, whilst the other animals had titres of between 1280 and 10240 (Figure 7.25E). There was no clear correlation between the magnitude of increase in ELISA titre following challenge and the development of secondary lesions, although rabbits that did develop secondary lesions upon challenge (282 and 288) had the highest increases in 50% PRNA titre (512-fold).

#### 7.2.5.5 Summary

Following inoculation with Uriarra  $\Delta$ M063Rgptgus, 12 week old rabbits showed a very similar reaction to mature rabbits, developing a small lesion at the inoculation site only, that was completely resolved by 21 dpi. There was very little anti-myxoma virus antibody (ELISA titre  $\leq$  100) measured in rabbits following the first dose of vaccine. The response upon boosting was similar to that seen during the initial vaccination, although the lesion at the inoculation site developed and regressed more rapidly. All rabbits showed significant increases in anti-myxoma virus antibody titres following boosting.

Uriarra  $\Delta$ M063Rgptgus-boosted rabbits showed very high levels of resistance to challenge with SLS, exhibiting clinical signs similar to those seen in rabbits vaccinated with TKO. Infection was limited to a lesion at the challenge inoculation site only on four rabbits, while two rabbits developed five small secondary lesions each on the face or ears. Following

challenge, all animals showed large increases (16-fold or greater) in anti-myxoma virus antibody titres.

## 7.2.6 Boosting Uriarra $\Delta$ M063Rgptgus-vaccinated rabbits with TKO

### 7.2.6.1 Boosting rabbits with TKO

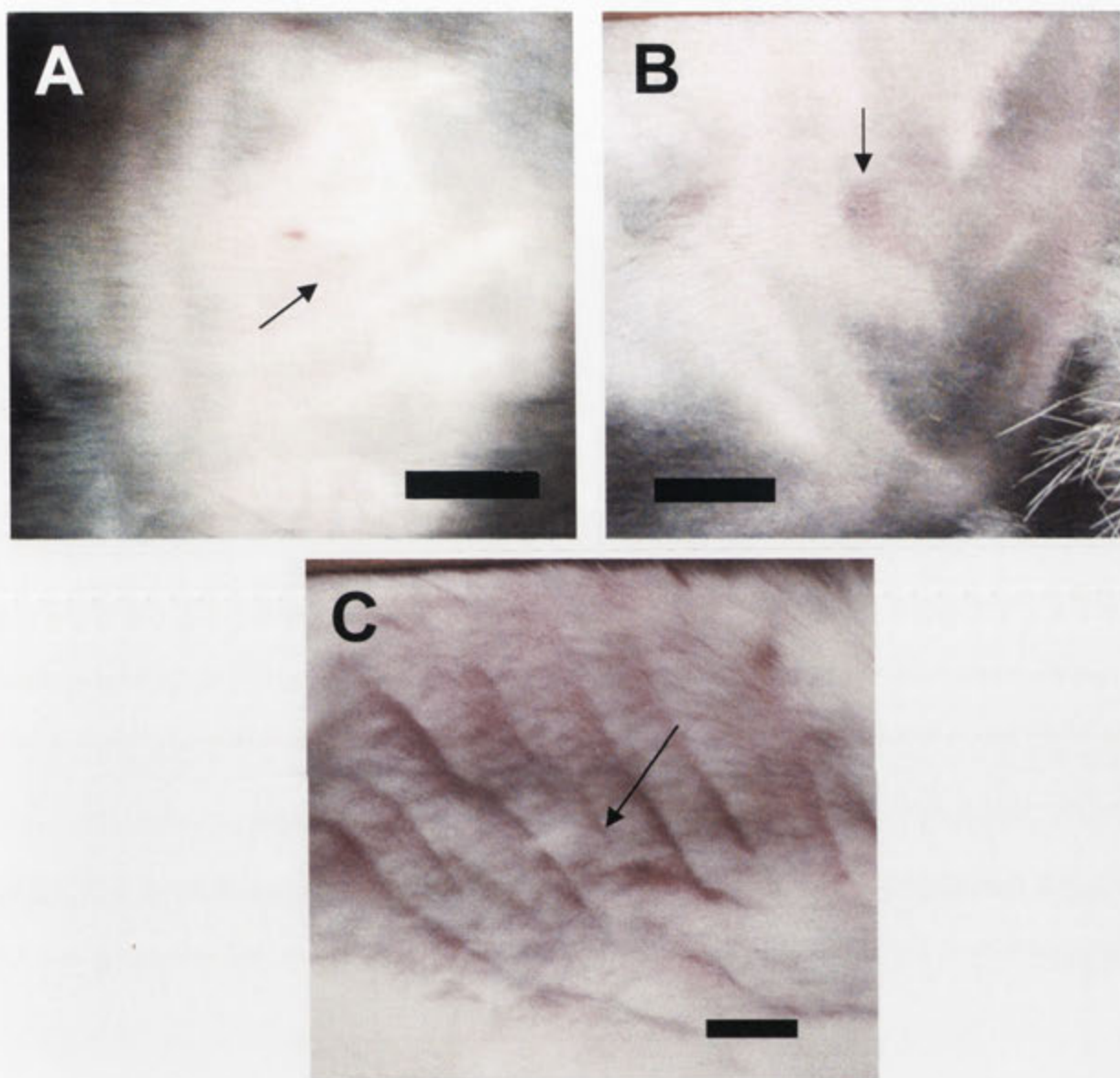
Four weeks after inoculation with Uriarra  $\Delta$ M063Rgptgus (Section 7.2.5.1), six of the 12 rabbits were boosted with 50 pfu of TKO intradermally on the right thigh and examined daily for 10 days. The clinical signs of these rabbits following boosting are summarised in Table 7.4. Unlike rabbits boosted with Uriarra  $\Delta$ M063Rgptgus, there was not an immediate reaction at the inoculation site on all rabbits. By 3 dpb, five rabbits had developed a slight reddening at the inoculation site or a small, soft pink lesion (Figure 7.26A and B) and one had no observable reaction (Figure 7.26C). This last rabbit began to develop a small lesion at 4 dpb. By 6 dpb, the slight reddening of the skin of TKO-boosted rabbits had developed into a small red or pink lesion, 5-15 mm in diameter on most rabbits (Figure 7.27A-C). The diameter of the lesion at the inoculation site at 6 days after boosting with TKO (mean = 10.7 mm) was significantly lower than seen on naive 12 week old rabbits inoculated with 50 pfu of TKO (mean = 17.3 mm;  $p = 0.177$ ). The exception was rabbit 290, which had developed a larger 20x30 mm lesion, which was covered by a hard black scab (Figure 7.27D). When this animal was excluded from the data set, the difference in TKO-induced lesion diameters was more significant (mean = 5.52 mm;  $p < 0.01$ ).

By 10 dpb, the lesion had regressed completely on three animals (285, 293 and 275) or had scabbed over on two others (289 and 291). The larger lesion on rabbit 290 had also begun to regress slightly, and was covered with a hard black concave scab. No secondary lesions

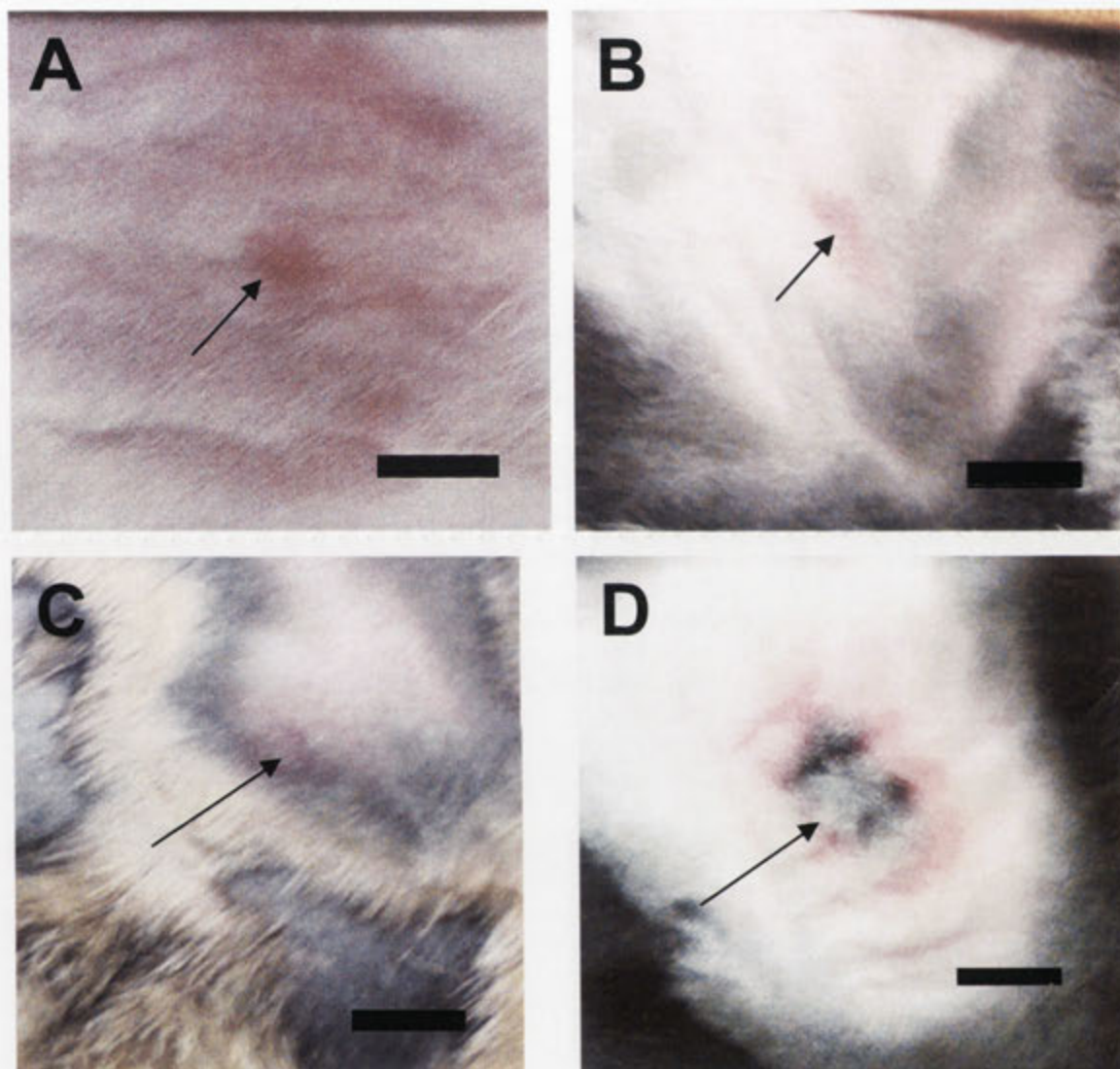
**Table 7.4** – Clinical signs of Uriarra ΔM063R*gptgus*-vaccinated rabbits following boosting with 50 pfu of TKO.

Day post inoculation	Clinical signs
1	Slight reddening of skin at the inoculation site of rabbit 293 and 290 only
3	5-8 mm raised pink lesion on 5 rabbits. Rabbit 275 had no reaction at the inoculation site.
5	Lesion at the inoculation site of 5/6 rabbits, crimson-coloured, 6-25 mm diameter. Darker patches on lesion of rabbit 290. Lesion on rabbit 285 had regressed.
7	Lesion of rabbit 290 was 20x30 mm, covered with a hard black scab. Other lesions were 5-15 mm in diameter, hard crimson lumps. No lesions on rabbits 285 and 275.
10	No lesion on rabbits 285, 275 or 293. A scabbed over, protuberant lesion (8-30 mm) on rabbits 290, 291 and 289. No secondary lesions on any animal.





**Figure 7.26** - Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits boosted with 50 pfu of TKO, 3 days after inoculation. Reddening of the skin at the inoculation site as shown (A and B, arrowed). No reaction at the inoculation site on one rabbit (C, arrowed). Bars on images are 10 mm in size.



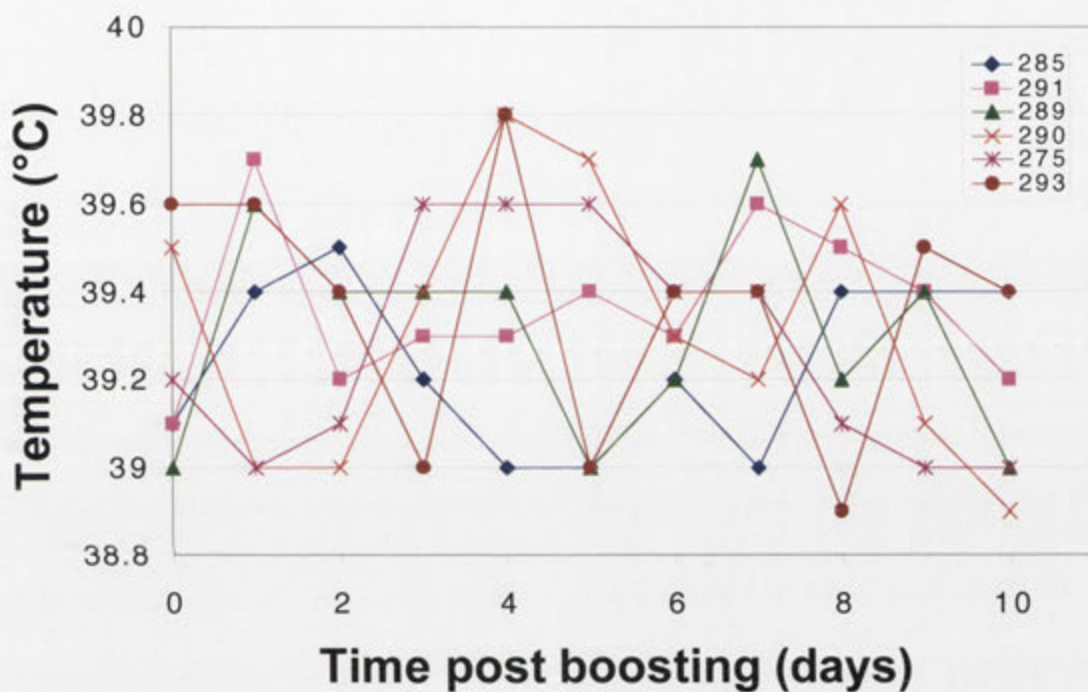
**Figure 7.27** - *Uriarra ΔM063Rgptgus* vaccinated rabbits boosted with 50 pfu of TKO, 6 days after inoculation. Lesions that had regressed to a small pink or red lump (A and B, arrowed). Larger (10-30 mm) lesions that were circumscribed or scabbed over (C and D, arrowed). Bars on images are 10 mm in size.

were noted on any rabbit following inoculation with TKO. The rectal temperatures of TKO-boosted rabbits remained below 40°C for the 10 days following boosting (Figure 7.28).

#### 7.2.6.2 Challenge of TKO-boosted rabbits with SLS

The six TKO-boosted rabbits were challenged with 1000 pfu of SLS at a site on the back above the left thigh 6 weeks after boosting, and examined for 14 days following challenge. The clinical symptoms of these animals are summarised in Table 7.5. Within 24 hours of SLS inoculation, a 5-15 mm pink or red raised lesion had developed at the inoculation site which had increased in size to 20-35 mm by 3 dpc, and were dark red in colour with black haemorrhagic regions on three of the rabbits (Figure 7.29). At 4 dpc, small secondary lesions began to develop on two rabbits (289 and 285). By 5 dpc, three rabbits (285, 293 and 289) had very large lesions at the inoculation site, in excess of 30 mm, which were highly protuberant and haemorrhagic, and these rabbits had also developed small (5-10 mm) crimson secondary lesions on the eyelids, genitals, ears and face. One other rabbit (275) had developed small (5 mm) black secondary lesions on the lips and ears.

By 6 dpc, the infection in one rabbit (290) had almost resolved, with a black scab covering a 20 mm lesion at the inoculation site (Figure 7.30C), and no secondary lesions evident. Two other rabbits (291 and 275) only had only 15-20 mm primary lesions (Figure 7.30A) and rabbit 275 had developed several very small secondary lesions. These animals had recovered by 9 dpc, except for black scabs at the inoculation site and secondary lesions (Figure 7.31A and B). The other three rabbits had lesions at the inoculation site 40-55 mm in diameter (Figure 7.30B, rabbit 293) and numerous secondary lesions on the eyelids, ears, body, nose and genitals (Figure 7.30D-H). The secondary lesions on these rabbits were

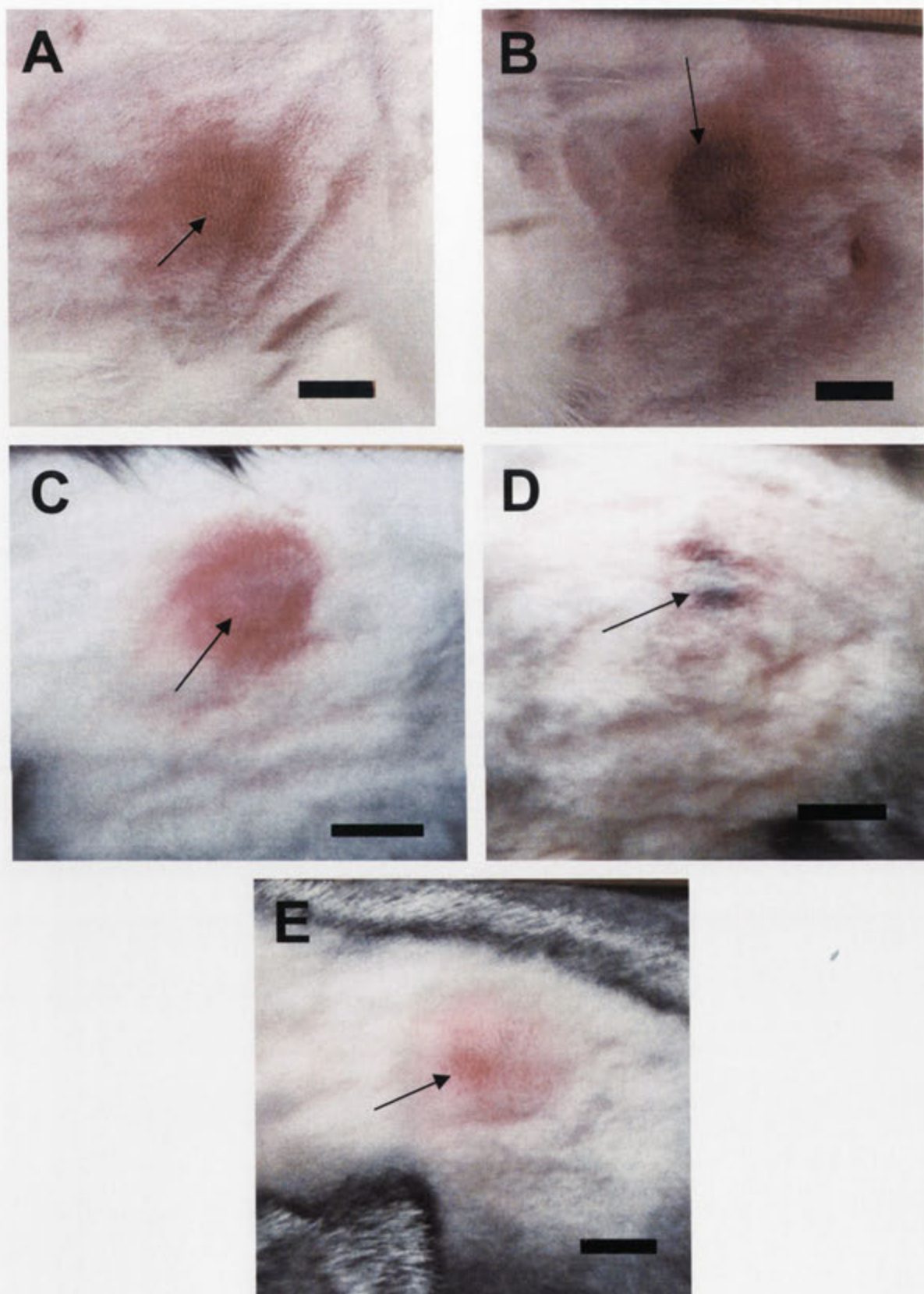


**Figure 7.28** - Rectal temperatures of Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits following boosting with 50 pfu of TKO.

**Table 7.5** – Clinical signs of TKO-boosted rabbits following challenge with SLS.

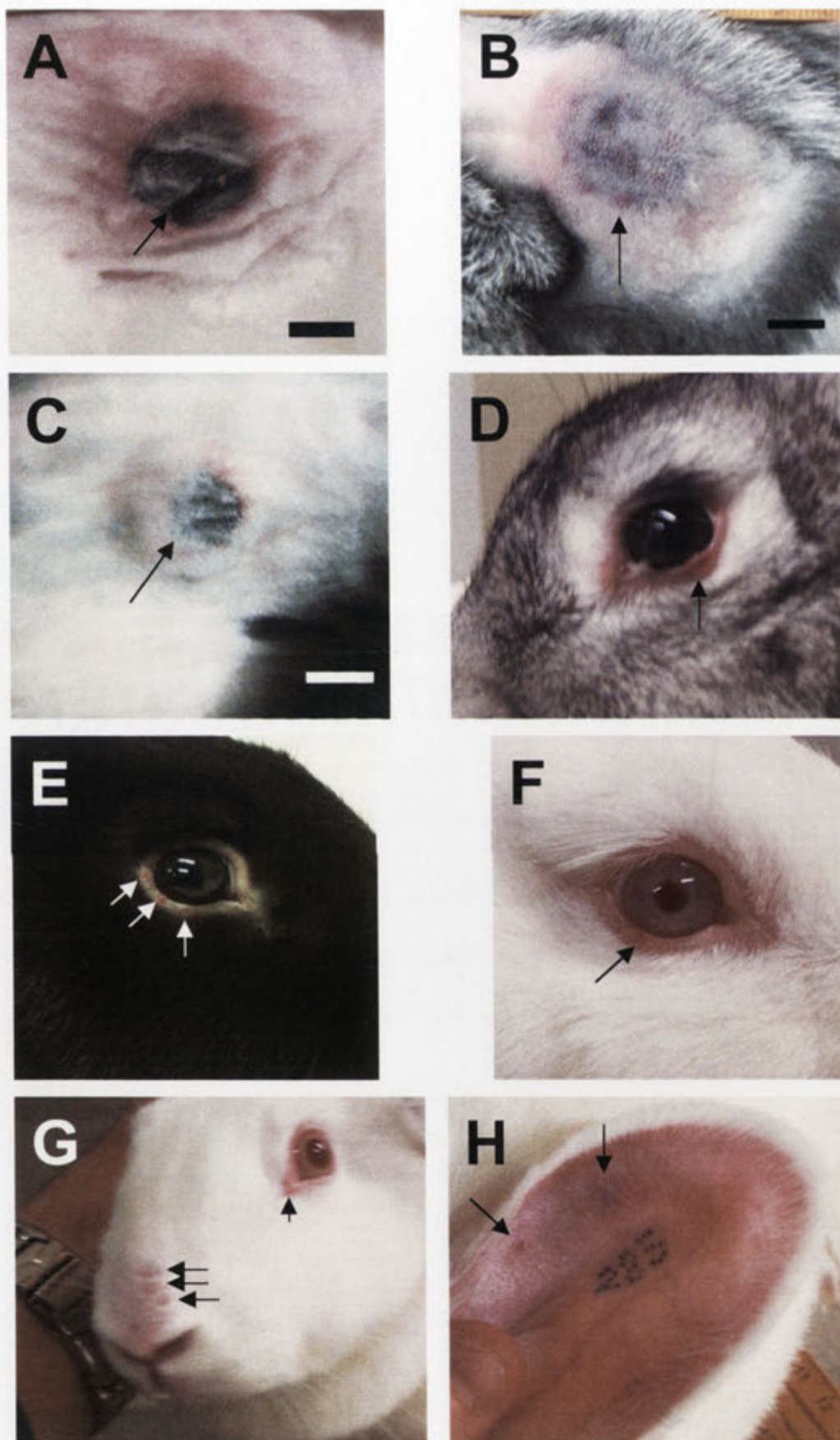
<b>Day post inoculation</b>	<b>Clinical signs</b>
<b>1</b>	Pink lesion at the inoculation site, 5-15 mm diameter
<b>3</b>	Red, protuberant lesion 20-35 mm in diameter; black, haemorrhagic patches on lesions of 3 rabbits. 2/6 rabbits had temperatures of >40°C.
<b>4</b>	Lesions at the inoculation site 21-35 mm diameter; black scab on lesion of rabbits 291 and 290. Secondary lesions on eyelid or ears of rabbits 289 and 285. 2/6 rabbits had temperatures of >40°C.
<b>5</b>	Lesion at the inoculation site of rabbit 290 regressing, covered with a hard black scab. Lesions of rabbits 291 and 275 circumscribed, scabbed over. Lesions at the inoculation site of rabbits 285, 289 and 293 were 25-35 mm diameter, protuberant, black and haemorrhagic. Secondary lesions on 4/6 rabbits on ears, eyelids, nostrils, anus, mouth. 4/6 rabbits had temperatures of >40°C.
<b>7</b>	Rabbit 290 and 291 recovered. Rabbits 275 had only a few small, black secondary lesions. Lesions at the inoculation site of rabbits 285, 289 and 293 were 40-60 mm in diameter, highly protuberant, bleeding or haemorrhagic. Numerous secondary lesions at secondary sites, mild mucopurulent discharge from conjunctivae, mild anogenital swelling. 3/6 rabbits had temperatures of >40°C.
<b>8</b>	As at day 7, except rabbits 289 and 293 had developed snuffly breathing and mucopurulent discharge from the nose. Lesions at the inoculation site covered with a thin black scab; lesion of rabbit 289 was bleeding. Secondary lesions covered by black scabby material except for on rabbit 293 - lesions still crimson and swollen. 3/6 rabbits had temperatures of >40°C.
<b>10</b>	Rabbit 285 recovering, though still had slight mucopurulent discharge from conjunctivae; no anogenital swelling. Breathing of rabbit 293 clear. Rabbit 289 had obstructed breathing and most secondary lesions were not black; anogenital swelling apparent. 2/6 rabbits had temperatures of >40°C.
<b>12</b>	Lesions at the inoculation site covered with hard black scabs. Rabbit 289 had recovered since day 10, though airways still slightly obstructed; other symptoms resolving; secondary lesions covered with black scabs; temperature still >40°C.
<b>15</b>	All rabbits recovered. Rabbits 285, 293 and 289 appear 'ratty' with lumpy, misshapen ears, and numerous black scabs on body and face.





**Figure 7.29** - Rabbits boosted with TKO, 3 days after challenge with 1000 pfu of SLS. Lesions at the inoculation site were red or crimson (A, C, and E, arrowed) or had developed black, haemorrhagic patches (B and D, arrowed). Bars on images are 10 mm in size.





**Figure 7.30** - Rabbits boosted with TKO, 6 days after challenge with 1000 pfu of SLS. Lesions at the inoculation site were covered with a hard black scab (A-C, arrowed) and up to 50 mm in diameter (B, arrowed). Secondary lesions on the eyelids (D-G, arrowed), nose (G, arrowed) and ears (H, arrowed). Bars on images are 10 mm in size.

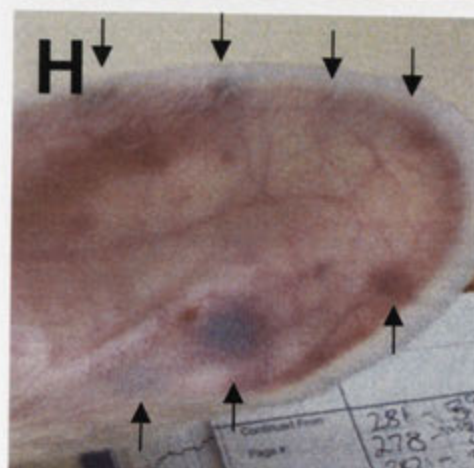
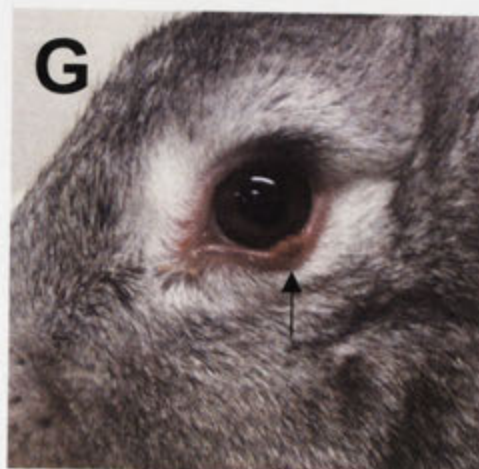
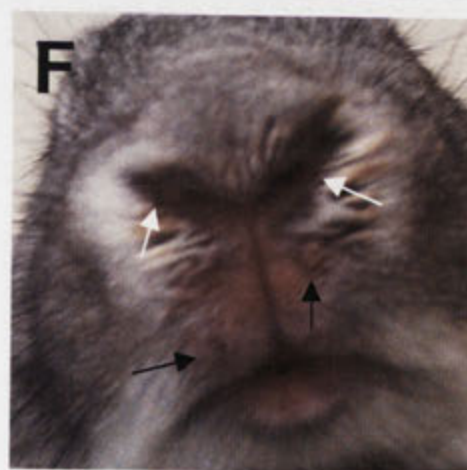
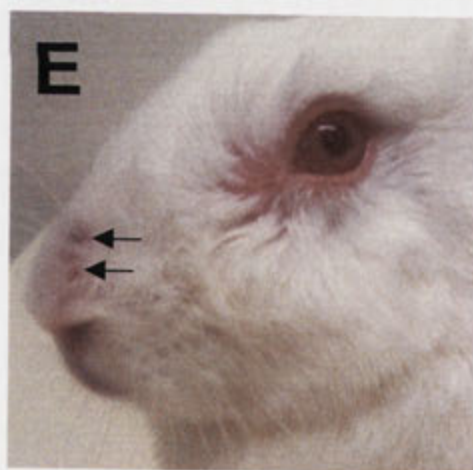
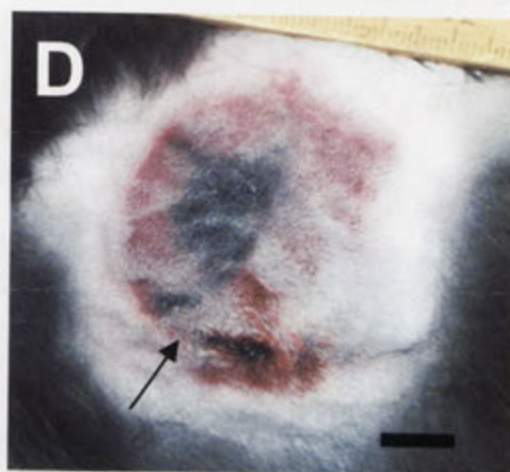
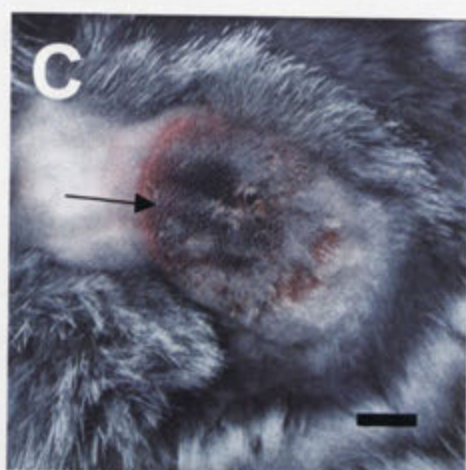
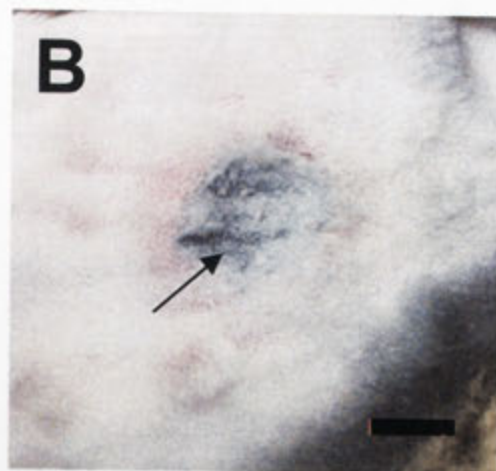
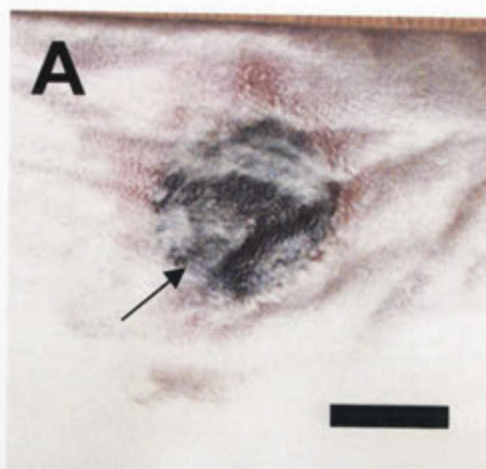
generally black in the centre and surrounded by crimson skin, except for rabbit 293 whose secondary lesions were red or crimson in colour with no black, haemorrhagic regions. Rabbits 289 and 293 had also developed a clear discharge from the conjunctivae and developed slightly snuffly breathing at 8 dpc.

By 9 dpc, the breathing of these two rabbits was more obstructed and the discharge from the conjunctivae and nostrils was thicker and mucoid, and lesions inside the nostrils were bleeding slightly (Figure 7.31F). The lesions at the inoculation site were circumscribed, highly protuberant, haemorrhagic and bleeding in places (Figure 7.31C and D). Rabbit 285 had begun to recover, although had lost some weight over the previous 3 days and had a clear discharge from the left eye (Figure 7.31E). Secondary lesions on the three more severely affected rabbits were black or haemorrhagic, although the surrounding skin was dark crimson in colour (Figure 7.31F-H). The breathing of rabbits 293 and 289 was normal at 10 and 13 dpc, respectively.

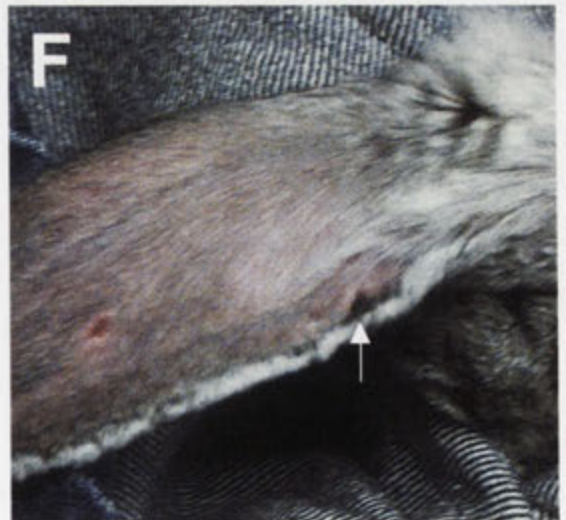
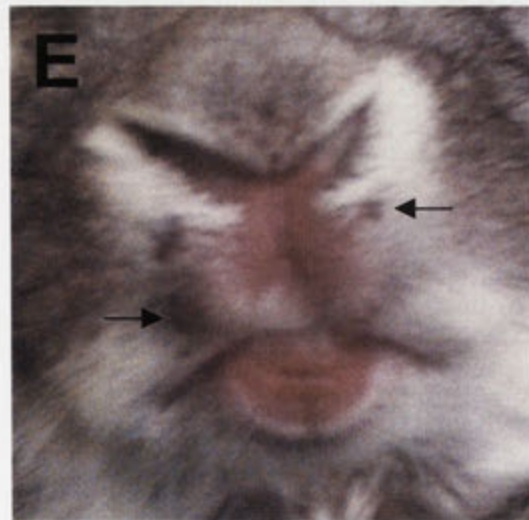
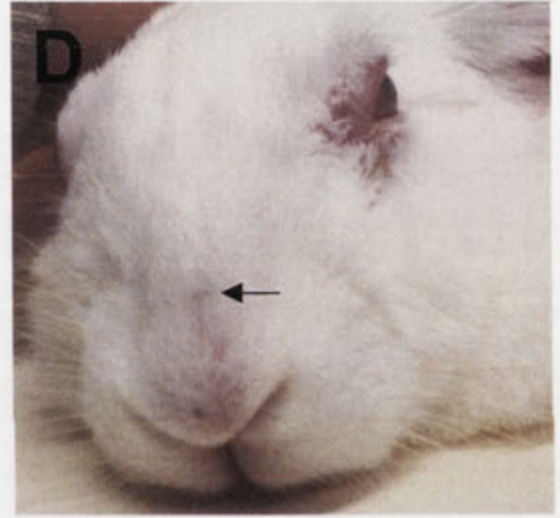
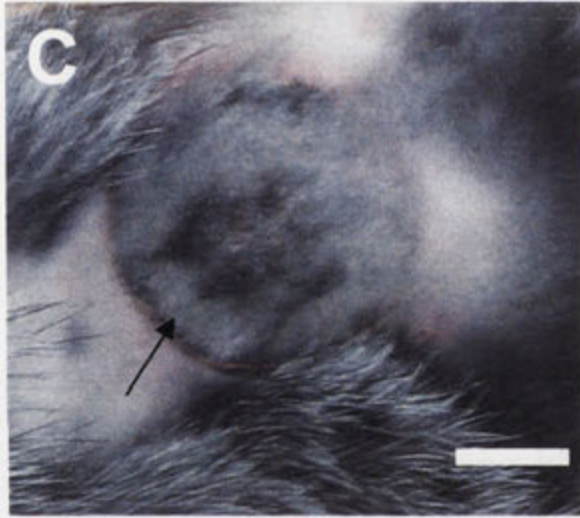
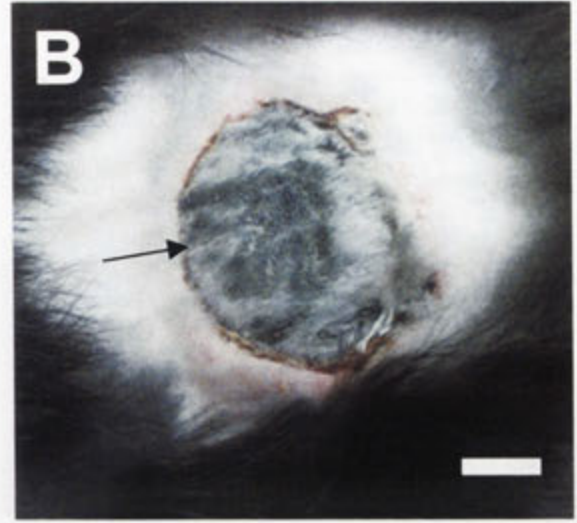
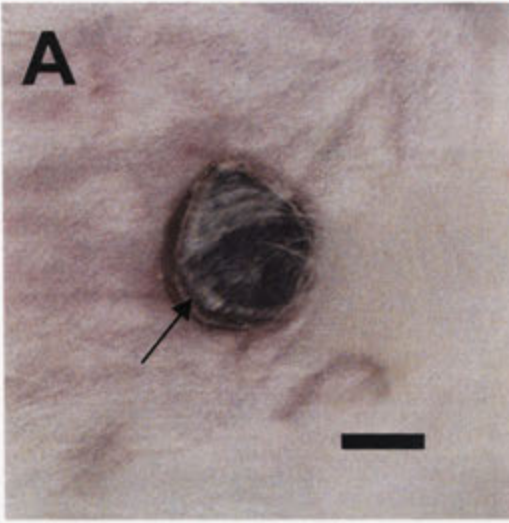
By 15 dpc all lesions at the inoculation site were covered with a hard black scab, and the surrounding skin had returned to normal (Figure 7.32A) or pale pink in colour (Figure 7.32B and C). Secondary lesions had regressed leaving black scabby material only (Figure 7.32D and E). The clearance of larger secondary lesions at the margins of the ears resulted in a moth eaten appearance to the edges of the ears due to the loss of pieces of skin (Figure 7.32F).

Rectal temperatures of rabbits were recorded each day following challenge and are shown in Figure 7.33. Rabbits 291, 275 and 290 did not have temperatures over 40°C for more than 1 day following challenge. Rabbits 285, 293 and 289 that showed more severe

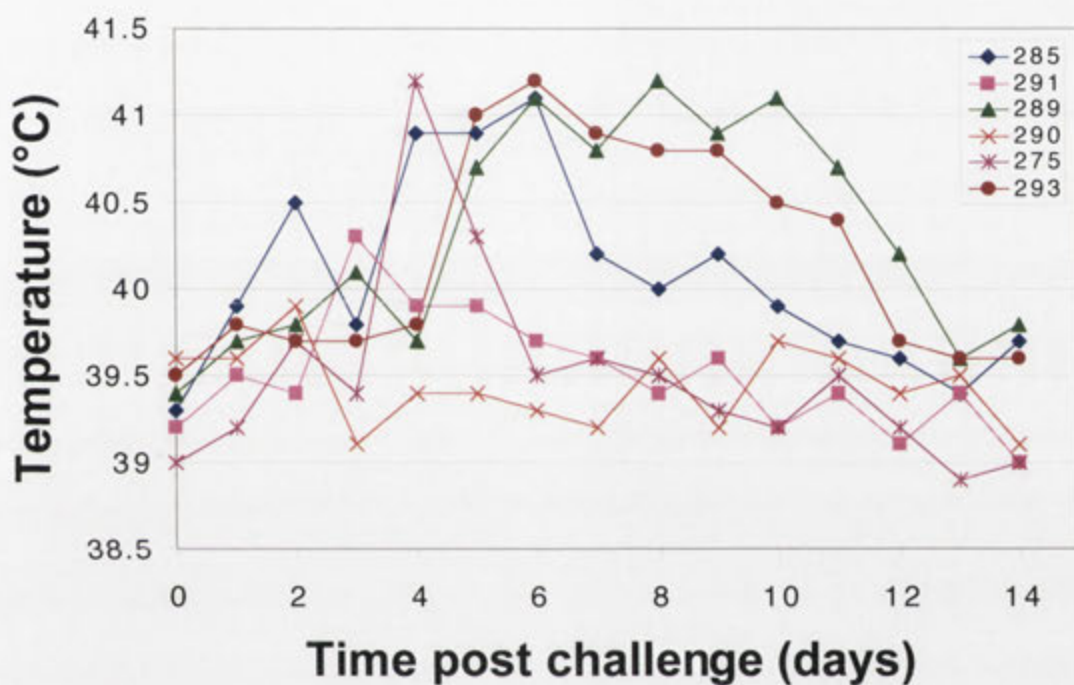
**Figure 7.31 - Rabbits boosted with TKO, 9 days after challenge with 1000 pfu of SLS.** Lesions at the inoculation site with residual black scab (A and B, arrowed). Very large lesions that were haemorrhagic and bleeding (C and D, arrowed). Secondary lesions on the nose (E and F, black arrows), eyes (G, arrowed) and ears (H, arrowed) were covered in scabby material, or haemorrhagic. Discharge and bleeding from the nostrils of rabbit 293 (F, white arrows). Bars on images are 10 mm in size.







**Figure 7.32** - Rabbits boosted with TKO, 15 days after challenge with 1000 pfu of SLS. Lesions at the inoculation site were covered by a residual scab (A-C, arrowed). Secondary lesions such as those on the nose and face (D and E, arrowed) had resolved to black scabs. A secondary lesion on the ear of rabbit 293 resulted in the loss of a 4 mm piece of tissue from the edge of the ear (F, arrowed). Bars on images are 10 mm in size.



**Figure 7.33** - Rectal temperatures of Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits boosted with TKO, following challenge with 1000 pfu of SLS.



symptoms had temperatures over 40°C for 7, 9 and 9 days respectively, including several days above 41°C.

In summary, two of the TKO-boosted rabbits (290 and 291) did not develop secondary lesions, including rabbit 290 which had developed the largest lesion following boosting with TKO (Figure 7.27D). One rabbit (275) developed only a few very small secondary lesions on the ears and eyelids following challenge. The symptoms of these three rabbits following SLS challenge were similar to animals that had received a single dose of TKO (Chapters 4 and 6) or had received two doses of Uriarra  $\Delta$ M063Rgptgus (Section 7.2.5.3). Three rabbits (293, 289 and 285) developed numerous, small secondary lesions on the eyelids, anus, nose, face or ears as well as more serious complications of myxomatosis such as mild laboured breathing, mild anogenital swelling, fever and some weight loss. However, at no point were these three rabbits seriously debilitated, being moderately ill for no more than 6 days. The symptoms exhibited by these rabbits were comparable to those exhibited by rabbits that had received a single dose of Uriarra  $\Delta$ M063Rgptgus (Section 7.2.4.2).

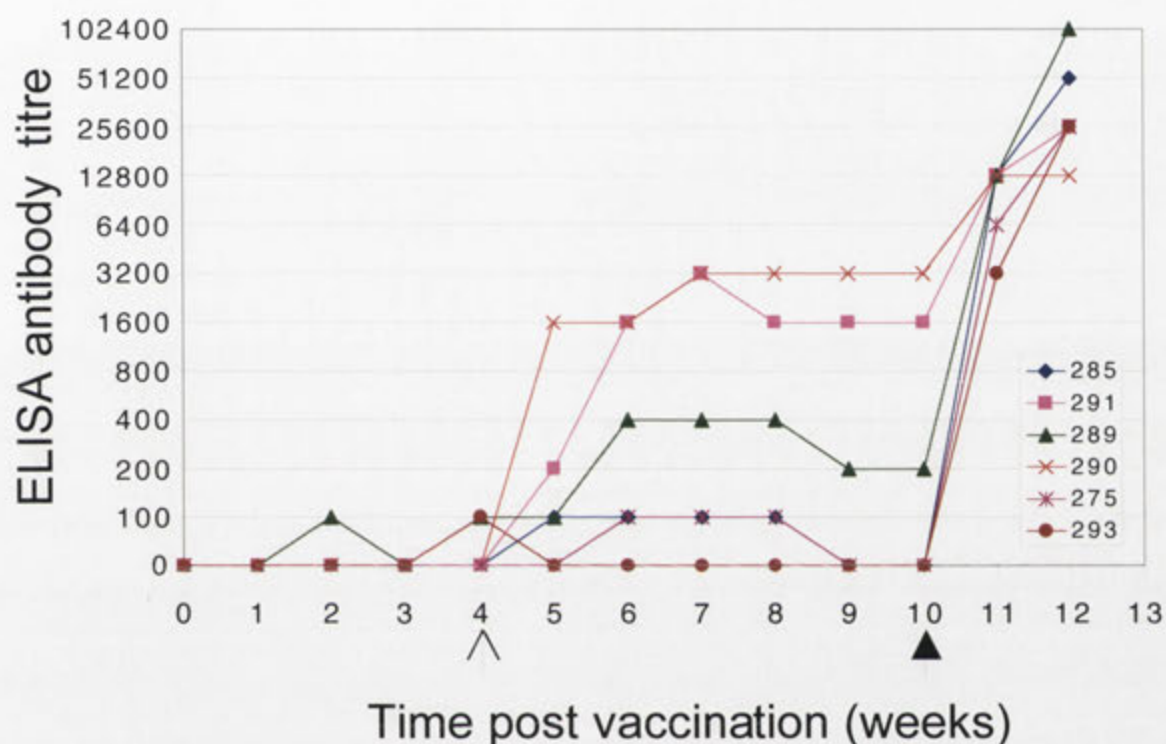
#### 7.2.6.3 Analysis of anti-myxoma virus antibody responses in TKO-boosted rabbits

The anti-myxoma virus ELISA antibody titre was determined for all serum samples after vaccination, boosting and challenge, and are shown in Figure 7.34. Antibody capable of neutralising myxoma virus *in vitro* was analysed using PRNA for serum samples taken before the TKO boost (4 wpv), 2 weeks after boosting (6 wpv), before challenge (10 wpv) and 2 weeks after challenge (12 wpv). The percentage plaque neutralisation for each serum dilution tested and the corresponding 50% and 80% plaque neutralisation titres are shown for each rabbit in Figure 7.35.

As was found with other groups of rabbits, only low titres of anti-myxoma virus antibody were detected following inoculation with 100,000 pfu of Uriarra  $\Delta$ M063Rgptgus. Only two rabbits (289 and 293) had detectable titres (100) by 4 wpv (Figure 7.34). PRNA titres were similarly very low. Only sera from rabbits 289 and 275 had any detectable virus-neutralising activity at 4 wpv (Figure 7.35A).

Following boosting with TKO, three rabbits (291, 289 and 290) had significant increases in ELISA titre over 2 weeks, with titres at 6 wpv of 1600, 1600 and 400 respectively (Figure 7.34). Rabbit 290, which developed the largest lesion following TKO inoculation, had a rapid increase in ELISA titre from <100 to 1600 within 7 days of boosting, before stabilising at 3200 at 7 wpv. Three rabbits (285, 275 and 293) did not have significant increases in antibody titre and by the time of challenge at 10 wpv, no anti-myxoma virus antibody could be detected in their serum by ELISA (Figure 7.34). Sera from rabbits 291, 289 and 290 also had increases in virus-neutralising activity between 4 and 6 wpv, with 50% PRNA titres of between 40 and 640 (Figure 7.35B). As with ELISA results, rabbit 290 had the highest 50% PRNA titre of 640 at 6 wpv (Figure 7.35E) falling to 320 at 10 wpv (Figure 5.35C and E). Rabbits 291 and 290 showed similar slight drops in titre by 10 wpv (Figure 5.35C and E). Sera from rabbits 285, 275 and 293 had very little virus-neutralising activity at 6 or 10 wpv (Figure 7.35B and C). In contrast, increases in ELISA and neutralising antibody titres were seen in all rabbits boosted with Uriarra  $\Delta$ M063Rgptgus (Figures 7.24 and 7.25), or challenged with SLS (Figures 7.13 and 7.14).

All TKO-boosted rabbits had significant increases (4-fold or greater) in ELISA and PRNA antibody titres following challenge with SLS at 10 wpv (Figures 7.34 and 7.35). Rabbits 291 and 290, which did not develop secondary lesions after challenge had smaller increases



**Figure 7.34** - ELISA anti-myxoma virus antibody titres in rabbits vaccinated with Uriarra  $\Delta$ M063Rgptgus and boosted with TKO. The ELISA titre was determined by titration in duplicate of two fold serial dilutions of rabbit sera from 1:100. The ELISA endpoint was defined as the reciprocal serum dilution 0.1 OD<sub>405</sub> units above the OD<sub>405</sub> of the 1:100 negative control. An OD<sub>405</sub> of less than 0.1 for a 1:100 dilution of serum was recorded as a titre of zero. At week 4, rabbits were boosted with 50 pfu of TKO (open arrow) and challenged with SLS at week 10 (closed arrow).

**Figure 7.35 - Analysis of neutralising anti-myxoma virus antibodies in the serum of rabbits vaccinated with Uriarra  $\Delta$ M063Rgptgus and boosted with TKO.** Neutralising antibody was measured by plaque reduction neutralisation assay and the percentage plaque neutralisation recorded before boosting (week 4, A), after boosting (week 6, B), before challenge (week 10, C) and after challenge (week 12, D) for each serum dilution (two-fold from 1:10 to 1:20480). The highest reciprocal serum dilutions at which 50% or greater and 80% or greater of myxoma virus plaque formation was neutralised is summarised (E).



than other rabbits (16- and 4-fold increases over 14 days, respectively), and their titres at the time of challenge were significantly higher than the other rabbits in the experimental group. Rabbits 285, 289 and 293 which developed the most severe symptoms of myxomatosis following SLS challenge showed very large increases in titre in the two weeks following challenge (1024-, 512- and 512-fold increases respectively), and had titres ranging from 25600 to 102400 (Figure 7.34). Rabbit 289, which had exhibited the most severe symptoms following challenge had virus neutralisation levels of 86% at a serum dilution of 1/20480 (Figure 5.35D) and an ELISA titre of 102400, the highest recorded anti-myxoma virus antibody titres in this study. All rabbits in this group had ELISA titres in excess of 12800 (Figure 7.34) and 50% PRNA titres of between 640 and >20480 at 12 wpv (Figure 7.35D and E).

#### 7.2.6.4 Summary

Boosting with TKO did not result in consistent responses amongst Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits. Responses ranged from a transient reddening at the inoculation site to a large protuberant lesion similar to that which developed on naive rabbits (Chapters 4 and 6). Changes in anti-myxoma virus antibody responses after boosting were directly related to the response upon boosting, with rabbits that developed a distinct TKO-induced lesion showing larger increases in antibody titres.

Following SLS challenge, TKO-boosted rabbits could be divided into two groups. Three rabbits did not develop symptoms of myxomatosis, with infection limited to a primary lesion and/or several small secondary lesions, similar to that seen in TKO-vaccinated and Uriarra  $\Delta$ M063Rgptgus-boosted rabbits. These rabbits had developed a distinct lesion at the inoculation site upon boosting with TKO. The other three rabbits developed mild

clinical myxomatosis that was similar to that observed in some rabbits that had received a single dose of Uriarra  $\Delta$ M063Rgptgus. These rabbits did not develop a distinct lesion upon boosting with TKO, or had developed a small, rapidly resolved lesion. All TKO-boosted rabbits had significant increases in anti-myxoma virus antibody titres following challenge, with the magnitude of the increase directly related to the severity of symptoms observed upon challenge.

### 7.3 DISCUSSION

In this chapter I have described the construction and testing of a replication-deficient myxoma virus vaccine, Uriarra  $\Delta$ M063Rgptgus. This is the first reported evaluation of the protective efficacy of a homologous, replication-deficient vaccine against a lethal poxvirus disease outside of Orthopoxvirus models, and has demonstrated the efficacy of a replication-deficient, and therefore non-transmissible myxoma virus vaccine against myxomatosis. Uriarra  $\Delta$ M063Rgptgus conferred complete protection against lethal myxoma virus challenge, and good protection against clinical disease. When boosted with another dose of Uriarra  $\Delta$ M063Rgptgus, rabbits had greater resistance to lethal SLS challenge, comparable to that seen in TKO-vaccinated rabbits. Boosting with TKO did not consistently offer greater levels of protection than a single dose of Uriarra  $\Delta$ M063Rgptgus.

The growth and protein expression characteristics of the virus, the acceptability and efficacy of Uriarra  $\Delta$ M063Rgptgus vaccination compared to replication-competent vaccines, and the role of virus-specific antibody and cell-mediated immune responses in protecting against SLS challenge have been discussed below.



### 7.3.1 Growth and protein expression characteristics of Uriarra $\Delta$ M063Rgptgus

Uriarra  $\Delta$ M063Rgptgus was unable to replicate within RK13 cells *in vitro*, suggesting that this recombinant virus would have similarly poor replicative ability in other rabbit cell lines and rabbit cells *in vivo*, as demonstrated by McFadden *et al* (personal communication). However, unlike the results of McFadden *et al.*, we have demonstrated readily detectable expression of an intermediate or late gene in Uriarra  $\Delta$ M063Rgptgus-infected RK13 cells using the monoclonal antibody 3B6E4. The specific target of this antibody has not been determined. Expression of the target protein was not detected in myxoma virus-infected cells at 4 hours after infection, but was detected at 6 hours after infection, and expression of this protein was prevented in infected cells treated with cytosine arabinoside, an inhibitor of DNA synthesis (Peter Kerr, personal communication). These two results suggest that the target protein is an intermediate or late gene, whose expression occurs during or after viral DNA replication (Moss, 1990; Moss, 2002). However, despite the low-level expression of this gene and presumably other intermediate and late genes, new virus is not produced, indicating that the replication cycle of Uriarra  $\Delta$ M063Rgptgus in RK13 cells is aborted at some stage before virus assembly.

The expression of late genes in Uriarra  $\Delta$ M063Rgptgus-infected rabbit cells, without associated production of progeny virus, allows a broader range of myxoma virus antigen production *in vivo* and a greater array of antigenic epitopes to be exposed to the rabbit's immune system than if virus gene expression is limited to early genes only. There is currently little information available regarding the protective antigens of poxviruses, so the ability of this virus to express as diverse a range of viral antigens as possible, without concurrent viral replication, will enhance its potential immunogenicity and efficacy.

### 7.3.2 Acceptability and efficacy of Uriarra $\Delta$ M063Rgptgus vaccination and boosting

Vaccination with Uriarra  $\Delta$ M063Rgptgus was much more acceptable than vaccination with the replication-competent vaccines tested, including the highly attenuated TKO vaccine, where the primary lesion haemorrhages and leaves a hard black scab at the inoculation site. In contrast, the lesion induced by Uriarra  $\Delta$ M063Rgptgus, both during the first vaccination and after boosting, was small and resolved very quickly, often without residual scabbing. The uniformity of responses and reactions in mature and 12 week old rabbits following Uriarra  $\Delta$ M063Rgptgus vaccination suggests that this dose of virus would be similarly acceptable in rabbits younger than 12 weeks old. However, the long-term effects of scarring at the inoculation site requires further investigation. As Uriarra  $\Delta$ M063Rgptgus is incapable of dissemination, it cannot cause secondary lesions and possible scarring at distal skin sites such as the ears, as may occur following TKO vaccination of younger rabbits (Chapter 6).

The reaction at the inoculation site of Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits was seen 2-3 days later than following inoculation with a 50-fold lower dose of TKO. The timing of these changes coincide with the appearance of antigen-specific CTLs observed in the draining lymph node during VV infections in rats (Zinkernagel *et al.*, 1977) and sheep (Issekutz, 1984; Issekutz, 1985), suggesting that the observed reaction was due to an influx of virus-specific lymphocytes into the skin with associated inflammation, rather than the virus-induced tissue damage and inflammation caused by a replication-competent virus.

Uriarra  $\Delta$ M063Rgptgus conferred excellent levels of protection from SLS challenge, with only a third of rabbits developing more than very mild secondary signs of infection. Although the symptoms exhibited these animals were more severe than preferred for an

‘ideal’ vaccine, it is unlikely that rabbits would succumb to SLS challenge. The symptoms were very mild compared to infection of naive rabbits with SLS (Best and Kerr, 2000; Robinson *et al.*, 1999). Rabbits that developed the most severe manifestations of disease were still very well protected from challenge, and at no point were seriously debilitated, remaining active and alert at all times.

The efficacy of Uriarra  $\Delta$ M063Rgptgus was drastically improved by boosting, with much greater levels of protection than that observed in rabbits that had received only a single dose of the replication-deficient vaccine. This level of efficacy was comparable to a single inoculation of TKO, with only two of the six rabbits showing dissemination of the virus to secondary sites and all animals protected from any other manifestations of myxomatosis.

It would be interesting to investigate the effect of higher doses of Uriarra  $\Delta$ M063Rgptgus on the levels of antigen-specific responses induced, and the subsequent level of protective efficacy against SLS challenge. MVA has been delivered at doses of up to  $10^8$  pfu, and the induction of virus-specific immune responses (antibody and CD8<sup>+</sup> T cells) and corresponding protective efficacy against lethal VV challenge was dose-dependant (Wyatt *et al.*, 2004). The dose of Uriarra  $\Delta$ M063Rgptgus delivered ( $10^5$  pfu) was selected as it was the maximum achievable without also injecting large amounts of Vero cell debris, with unknown effects on the infection. The delivery of higher doses of Uriarra  $\Delta$ M063Rgptgus would require higher concentrations of the virus than those obtained during these studies. This could be achieved by ultracentrifugal purification of virus, the optimisation of virus growth conditions in Vero cells such as the use of roller bottles, or evaluating other cells lines that may be capable of producing higher titres of virus.

### 7.3.3 Efficacy of boosting with TKO

The dose of TKO used to boost rabbits in these experiments may have been too low to produce consistent boosting of anti-myxoma virus immune responses. It was selected as it was believed 50 pfu would reduce the incidence of dissemination of the vaccine virus to secondary sites in Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits, but would be high enough to provide a significant boost to immunity. TKO replication in most rabbits was limited at the inoculation site as indicated by the rapid circumscription of the lesion and its small size on most rabbits. TKO-induced lesion size and severity in Uriarra  $\Delta$ M063Rgptgus-vaccinated rabbits was significantly lower than seen on naive rabbits. Similarly, a reduction in live VV-induced lesion size and severity was observed in monkeys previously vaccinated with a single dose of MVA (Earl *et al.*, 2004).

The very rapid control of TKO replication resulted in low levels of antigen synthesis in most rabbits and poor boosting of anti-myxoma virus immune responses. Similarly, the level of protection from SLS challenge in TKO-boosted rabbits was not consistently as high as that observed in Uriarra  $\Delta$ M063Rgptgus-boosted animals, and was comparable to a single dose of Uriarra  $\Delta$ M063Rgptgus in mature animals. The only animals to be completely protected from dissemination of the challenge virus were those that developed large lesions after TKO boosting. However, similar levels of protection were observed in animals inoculated with TKO alone. This suggests that although previous vaccination with Uriarra  $\Delta$ M063Rgptgus resulted in a reduction in the severity of TKO-induced symptoms in some rabbits, these animals were not provided with a boost in protection from SLS challenge. Further evaluation of TKO boosting using larger doses of the virus is warranted, to determine if more consistent boosting of protection can be obtained without a concurrent increase in the severity of the TKO-induced lesion or dissemination to secondary sites.

### **7.3.4 Production of anti-myxoma virus antibody following vaccination and boosting, and the correlation with protection**

Inoculation with  $10^5$  pfu of Uriarra  $\Delta M063Rgptgus$  did not elicit high levels of anti-myxoma virus antibody as was found after inoculation with TKO. This low level of antibody may be due to two factors. Firstly, the replication-deficient nature of the vaccine results in much lower levels of antigen production *in vivo* than inoculation with replication-competent viruses. Secondly, the poor expression of late myxoma virus proteins including IMV and EEV membrane proteins (Cameron *et al.*, 1999; Moss, 2002), may have resulted in only weak antibody responses against these targets of virus neutralisation. The ELISA titres would be similarly lower as the antigen used to coat the ELISA plates was intact IMV particles purified by ultracentrifugation, therefore consisting primarily of exposed IMV antigens.

Boosting of Uriarra  $\Delta M063Rgptgus$ -vaccinated rabbits with another dose of the virus resulted in significant and rapid (within 7 days) increases in serum antibody titres. This indicated that all rabbits possessed myxoma virus-specific B cells primed by the first dose of vaccine that resulted in a potent secondary antibody response, as naive rabbits vaccinated with replication-competent viruses do not display detectable antibody before 10-14 dpi (Chapters 4 and 6; Best and Kerr, 2000; Fenner and Ratcliffe, 1965). In contrast, boosting with TKO did not consistently result in increases in antibody titre. Those rabbits which did show increases in antibody titre were those that had developed larger TKO-induced lesions, suggesting that a greater amount of TKO replication and a more robust secondary immune response had occurred. Clearly, the very low level viral replication in some rabbits was not sufficient to generate a potent secondary antibody response.

These results support the premise that circulating neutralising antibody does not play a significant role in protection, as there was very little present at the time of challenge in many animals. This suggests that Uriarra  $\Delta$ M063Rgptgus-vaccinated animals possessed a pool of primed virus-specific CTLs and memory T cells capable of rapid expansion and control of the infection within a few days of challenge. The rapid infiltration of lymphocytes into the inoculation site is evident by the immediate reaction observed (within 24 hours), and the swift circumscription and haemorrhaging of the lesion. Without an accurate means of measuring myxoma virus-specific cell mediated immune responses, we cannot determine and compare the magnitude of cell-mediated immune responses generated following inoculation with Uriarra  $\Delta$ M063Rgptgus and after boosting. However, the high levels of protection observed in the absence of neutralising antibody suggests that cell-mediated mechanisms generated following vaccination with the replication-deficient vaccine were primarily responsible for the initial control of the infection at the inoculation site, and protection from myxomatosis.

### 7.3.5 Conclusions

The results presented in this chapter indicate that the deletion of a single open reading frame from Uriarra was capable of generating a safe, acceptable and non-transmissible replication-deficient myxoma virus vaccine, that could protect domestic rabbits from myxomatosis following challenge with a virulent virus. A single dose of  $10^5$  pfu of the virus conferred high levels of protection that were enhanced by a boost with the same vaccine. Although the symptoms exhibited by rabbits following challenge with SLS were more severe than observed in animals vaccinated with replication-competent vaccines, none of the challenged animals exhibited severe myxomatosis or were seriously debilitated. Together with the very mild reaction following inoculation and the non-transmissible



nature of this replication-deficient vaccine virus, Uriarra  $\Delta$ M063Rgptgus can be considered an excellent candidate vaccine for further development.

## **Chapter 8**

### **General discussion**

## CHAPTER 8 – GENERAL DISCUSSION

This thesis has evaluated three poxvirus vaccination strategies using infection of European rabbits with myxoma virus as a natural poxvirus disease model. The key findings of this study were:

1. Intramuscularly injected DNA vaccines expressing selected myxoma virus IMV and EEV membrane antigens were not able to protect rabbits from lethal myxoma virus challenge;
2. Live, attenuated myxoma virus vaccines confer excellent protection from challenge, but can cause extremely mild secondary signs of infection in young rabbits, and can reach potentially transmissible titres in the skin of vaccinated rabbits;
3. Replication-deficient myxoma virus vaccines can confer protection from challenge, but require boosting to achieve protection equivalent to live attenuated vaccines.

Both TKO and Uriarra  $\Delta$ M063Rgptgus conferred excellent protection from lethal myxoma virus challenge. The acceptability and efficacy of these two vaccines with respect to the criteria for an "ideal" myxomatosis vaccine established in Section 1.6 will be discussed, as well as the role of antibody and cell-mediated immune responses in protection from myxoma virus challenge, and future directions for the development of these candidate myxomatosis vaccines for use in Australia.

### 8.1 A comparison of TKO and Uriarra $\Delta$ M063Rgptgus as myxomatosis vaccines

The acceptability of TKO and Uriarra  $\Delta$ M063Rgptgus can be gauged by comparing the pathogenesis of these two viruses in domestic rabbits. Figure 8.1 shows a comparison of models for the pathogenesis of virulent myxoma virus (Best *et al.*, 2000; Best and Kerr,

**Figure 8.1 - A comparison of the models for the pathogenesis of SLS (A), TKO (B) and Uriarra  $\Delta$ M063Rgptgus (C) in domestic rabbits.**

**A:** Infection with SLS results in uncontrolled replication of the virus, dissemination to distal tissues and death. Before death, low levels of anti-myxoma virus neutralising antibody can be detected. Generalised immunosuppression caused by the virus inhibits the generation of virus-specific T cell responses that could otherwise control the infection.

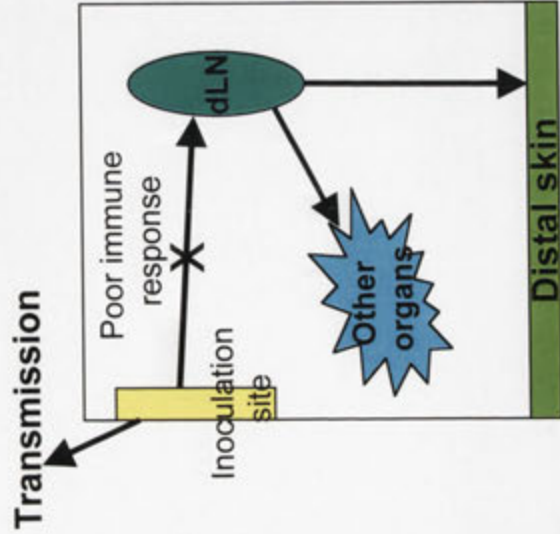
**B:** The loss of immunomodulatory genes in TKO results in an infection with limited ability to disseminate to distal tissues, and where more robust adaptive and innate immune responses can rapidly control the infection. Dissemination to distal skin sites occurs in the majority of 8 and 12 weeks old rabbits, but not in mature rabbits. The secondary lesions observed on younger rabbits may result in scarring. TKO virus at the inoculation site reaches titres that could result in transmission by mosquitoes. Following vaccination, rabbits had high levels of anti-myxoma virus antibody, and presumably, high levels of virus-specific T cells, inferred by the response at the inoculation site following challenge.

**C:** Uriarra  $\Delta$ M063Rgptgus cannot replicate or disseminate in rabbits. The infection of cells at the inoculation site results in the synthesis of viral antigens, which are likely transported to the draining lymph node where they are able to prime anti-myxoma virus T and B cells. However, only very low titres of anti-myxoma virus antibody are generated by a single dose of vaccine. Good levels of virus-specific T cells are generated, inferred by the rapid response at the inoculation site following challenge. The inability of the virus to replicate *in vivo* means that the virus cannot disseminate to distal tissues or transmit from the inoculation site.

Note a - Levels of virus specific T cells were not measured, but inferred based on rapid DTH-type responses seen at the inoculation site upon challenge.

# A

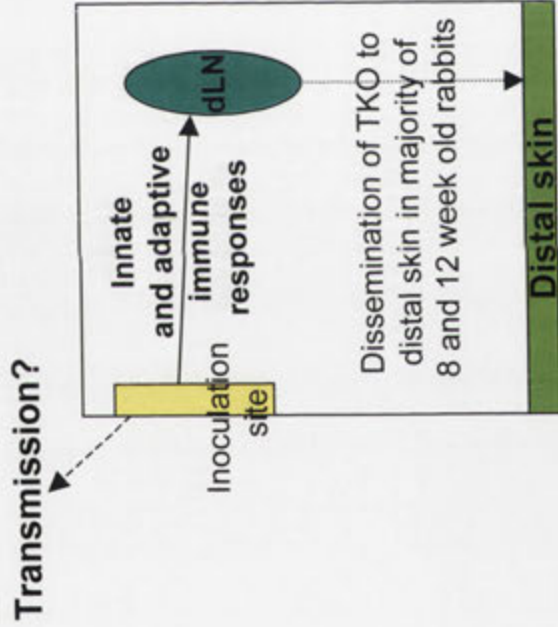
SLS



Uncontrolled infection  
DEATH

# B

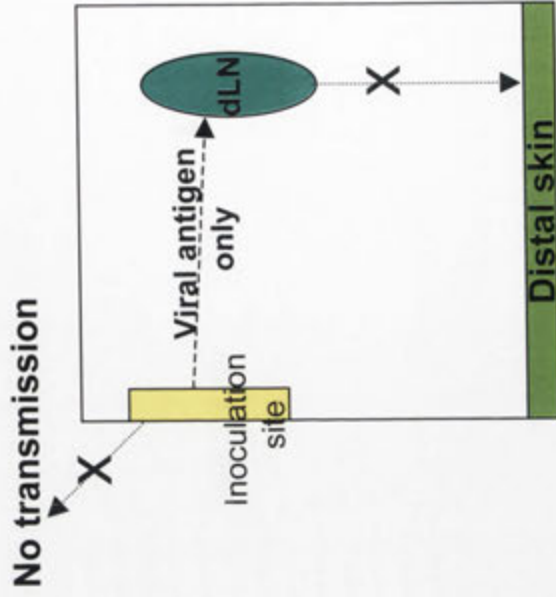
TKO



Virus cleared by 14-21 dpi  
Scarring in younger rabbits?

# C

Uriarra  $\Delta$ M063Rgptgus



Virus cleared by 10-14 dp

2000), TKO (Chapter 5) and the hypothesised pathogenesis of Uriarra  $\Delta M063Rgptgus$ . In contrast to the uncontrolled, lethal SLS infection (Figure 8.1A; Best and Kerr, 2000), infection with TKO is rapidly controlled. However, TKO can replicate to potentially transmissible titres in the skin of domestic rabbits, and disseminates to distal skin sites in a majority of 8 and 12 week old rabbits (Figure 8.1B). Although TKO appears to be safer than commercially available myxoma virus vaccines (Gorski *et al.*, 1994; McKercher and Saito, 1964; Saurat *et al.*, 1978), these results render the vaccine unacceptable for use in Australia according to the criteria established in Section 1.6.

Uriarra  $\Delta M063Rgptgus$  is the most acceptable vaccine for use in domestic rabbits. The inability of this virus to replicate and disseminate within the vaccinated rabbit means it cannot cause any secondary symptoms of myxomatosis or transmit to another animals (Figure 8.1C). However, in contrast to TKO vaccination, a single dose of Uriarra  $\Delta M063Rgptgus$  raises only low levels of virus-specific immune responses, presumably due to the comparatively low level of antigen synthesis *in vivo*. However, the rapid inflammatory response and large increases in anti-myxoma virus antibody seen immediately following boosting indicate that potent anti-myxoma virus memory (primed memory B and T cells) are generated by a single dose of Uriarra  $\Delta M063Rgptgus$ .

The efficacy of TKO and Uriarra  $\Delta M063Rgptgus$  vaccination against lethal myxoma virus challenge can be compared by considering the pathogenesis of SLS infection in vaccinated rabbits. Figure 8.2 compares the pathogenesis of SLS in TKO-vaccinated rabbits (Figure 8.2A), Uriarra  $\Delta M063Rgptgus$ -vaccinated rabbits (Figure 8.2B) and rabbits that were boosted with a second dose of Uriarra  $\Delta M063Rgptgus$  (Figure 8.2C). Vaccination with TKO induced moderate to high titres of anti-myxoma virus antibody and, most likely, high



**Figure 8.2 - A model for the pathogenesis of SLS infection in TKO-vaccinated (A), Uriarra *ΔM063Rgptgus*-vaccinated (B) and Uriarra *ΔM063Rgptgus*-boosted rabbits (C).**

**A:** In TKO-vaccinated rabbits, SLS replication was limited to the inoculation site in most animals, but SLS was able to disseminate to distal skin sites in 17% (6/36 rabbits) of TKO-vaccinated rabbits. Some rabbits showed an increase in anti-myxoma virus immune responses following challenge.

**B:** Due to the low level of anti-myxoma virus immune effectors (antibody and effector T cells) in Uriarra *ΔM063Rgptgus*-vaccinated rabbits, SLS replication at the inoculation site was not successfully controlled, and dissemination to distal tissues occurred in all animals, causing mild to moderate myxomatosis. The presence of robust memory B and T cell populations was evident by the very strong and rapid increases in anti-myxoma virus antibodies following challenge, and the rapid control of the infection, most likely by the proliferation of effector T cells from vaccine-induced memory populations. The virus replication at the inoculation site may result in transmissible titres of SLS. The vaccine conferred complete protection from death and severe myxomatosis, but not complete protection from disease.

**C:** SLS pathogenesis in Uriarra *ΔM063Rgptgus*-boosted rabbits was very similar to that in TKO-vaccinated rabbits, with limited dissemination of the challenge virus, and rapid control of the virulent infection. Most rabbits showed a strong increase in levels of anti-myxoma virus immune responses following challenge.

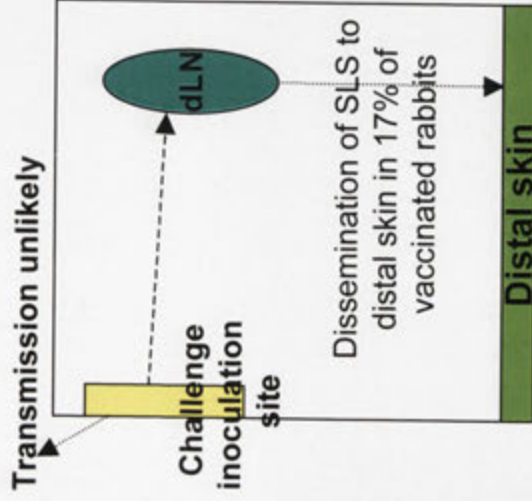
Note a - Levels of virus specific T cells were not measured, but inferred based on rapid DTH-type responses seen at the inoculation site upon challenge.

# A

TKO-vaccinated

Virus-specific immune responses present before challenge:

Antibody +++  
T cells<sup>a</sup> +++



**EXCELLENT PROTECTION FROM DISEASE**

Changes in immune responses after challenge:

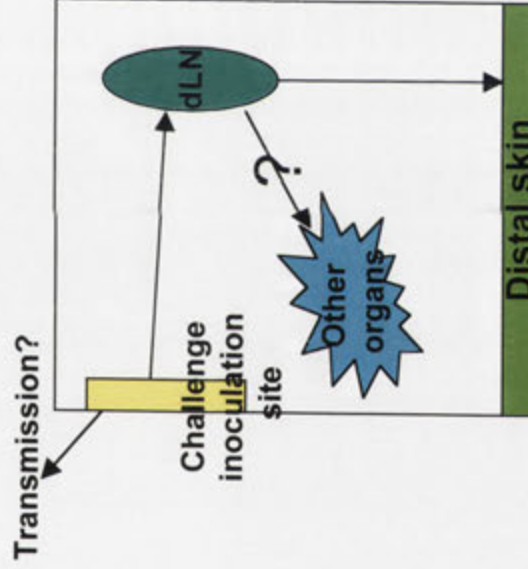
Antibody No change / ↑  
T cells<sup>a</sup> No change / ↑

# B

Uriarra ΔM063Rgptgus-vaccinated

Virus-specific immune responses present before challenge:

Antibody +/-  
Primed B cells +++  
Effector T cells<sup>a</sup> +/-  
Memory T cells<sup>a</sup> +++



**GOOD PROTECTION FROM DISEASE  
COMPLETE PROTECTION FROM DEATH**

Changes in immune responses after challenge:

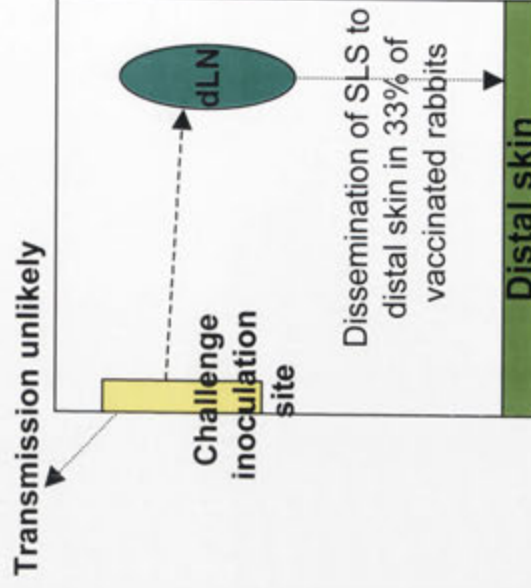
Antibody ↑↑↑  
T cells<sup>a</sup> ↑↑

# C

Uriarra ΔM063Rgptgus-vaccinated and boosted

Virus-specific immune responses present before challenge:

Antibody ++  
T cells<sup>a</sup> +++



**EXCELLENT PROTECTION FROM DISEASE**

Changes in immune responses after challenge:

Antibody ↑↑  
T cells<sup>a</sup> ↑↑

levels of anti-myxoma virus T cells (Figure 8.2A), conferring excellent protection from challenge. In contrast, a single dose of Uriarra  $\Delta$ M063Rgptgus, although conferring complete protection from death, did not prevent the dissemination of the challenge virus and the onset of mild to moderate myxomatosis (Figure 8.2B). This level of protection is better than that conferred by commercially available RFV-based vaccines (Marlier *et al.*, 2000). The protective efficacy of Uriarra  $\Delta$ M063Rgptgus is drastically improved by boosting (Figure 8.2C), with measured immune responses and post-challenge clinical signs similar to those of rabbits that had received a single dose of TKO (Figure 8.2A). Although the need for multiple doses of a vaccine to obtain protective immunity is economically undesirable for farmed rabbits, amongst companion animals, safety is considered of paramount importance, and multiple doses of vaccines or annual boosters are commonly administered (Carter and Carmichael, 2003).

The protective efficacy of Uriarra  $\Delta$ M063Rgptgus vaccination in the absence of anti-myxoma antibody indicates an important role for cell-mediated immune responses in protection from myxoma virus challenge. This is further supported by the rapid decline in protective efficacy seen following vaccination with a  $\Delta$ M063R myxoma virus strain, from complete protection at 2 weeks after vaccination (Grant McFadden, personal communication) to moderate protection at 6 weeks. Numbers of virus-specific CTLs decline by up to 95% within 2 weeks of virus clearance as virus-specific effector CTLs undergo apoptosis during the maturation of memory T cell populations (Esser *et al.*, 2003; Wherry and Ahmed, 2004), as observed in poxvirus disease models (Issekutz, 1984; Issekutz, 1985; Zinkernagel *et al.*, 1977). A rapid immune response was indicated by the early inflammation at the inoculation site of all animals following challenge. However, the population of effector T cells had contracted since two weeks after vaccination and were

not sufficient to completely control viral replication and dissemination, but controlled the virulent infection before the onset of severe myxomatosis. A second dose of the replication-deficient vaccine was able to boost anti-myxoma virus immune responses to the point where they were able to control virus replication at the inoculation site, with only limited challenge virus dissemination.

The results of this study suggest that replication-deficient poxvirus vaccines offer the best combination of safety and efficacy amongst the tested vaccination strategies. The efficacy of two doses of Uriarra  $\Delta$ M063Rgptgus was comparable to TKO and other live myxoma virus vaccines (Gorski *et al.*, 1994; Jacotot *et al.*, 1967; Marlier *et al.*, 2000; McKercher and Saito, 1964; Saurat *et al.*, 1978), with the added benefit of complete non-transmissibility and safety in younger rabbits. Replication-deficient poxvirus vaccines may be effective at combating veterinary poxvirus diseases of economic importance such as the capripoxviruses, orf and fowlpox. The targeted deletion of homologues of M063R and/or critical intracellular interferon response-resistance factors such as K1L and E3L (Alonso *et al.*, 2000; McInnes *et al.*, 1998; Tulman *et al.*, 2002), to produce homologous virus strains incapable of replication within the host may offer the best option for safer poxvirus vaccines.

The ability of live virus vaccines, whether replication-competent (with a single dose) or deficient (with multiple doses) to induce cell-mediated immune responses and neutralising antibody against a broad range of viral antigens offers the best chance of inducing long-term protective systemic immunity to poxvirus challenge. Further investigation using mouse models of poxvirus diseases may reveal vaccination strategies that are capable of conferring complete immunity from lethal poxvirus challenge through the induction of

narrowly targeted, specific immune responses, such as CD4<sup>+</sup>- or CD8<sup>+</sup>-restricted responses directed against single antigens or epitopes, or neutralising antibody directed against specific viral antigens. However, the restricted range of immune responses induced by such vaccination strategies in inbred mouse models is artificial, and does not take into account the diverse responses and immunogenetics of outbred populations.

## **8.2 Further development of Uriarra $\Delta$ M063Rgptgus for use as a myxomatosis vaccine in Australia**

From this study, the most promising candidate vaccine for use in Australia is the replication-deficient vaccine Uriarra  $\Delta$ M063Rgptgus. This vaccine possesses the optimal combination of acceptability and efficacy, and its replication-deficient nature eliminates the possibility of transmission to wild animals. Long-term efficacy trials are currently under way to evaluate how long protection from challenge is maintained after two doses of Uriarra  $\Delta$ M063Rgptgus. Further investigation and characterisation of this vaccine should include:

- Testing of higher doses of Uriarra  $\Delta$ M063Rgptgus and alternative delivery routes.

MVA is more immunogenic when delivered intramuscularly than intradermally (Munz *et al.*, 1993), while NYVAC is more effective when delivered intradermally (Belyakov *et al.*, 2003; Tartaglia *et al.*, 1992). The high density of professional antigen presenting cells in the dermis that can be directly infected by myxoma virus (Best *et al.*, 2000) may make this the optimal injection site, although it does limit the injection volume, and therefore the viral dose, that can be injected compared to intramuscular injection. As these cells are central to the induction of the immune response and the transport of viral antigens to the regional lymph node (Banchereau and Steinman, 1998), the early

infection of these cells may explain the efficacy of the replication-deficient vaccine despite overall lower levels of viral antigen synthesis.

- Effects of maternally transferred antibody on vaccine efficacy. Maternally transferred antibody can offer protection from lethal myxoma virus challenge to a small proportion of young rabbits (Fenner and Marshall, 1954), and is likely to interfere with the efficacy of a highly attenuated vaccine virus infection, preventing the induction of full protective immunity. Anti-myxoma virus antibody could not be detected by ELISA in young wild rabbits born to immune does by 6 weeks after birth (Kerr, 1997) or by PRNA and complement fixation assay at 7 weeks after birth (Fenner and Marshall, 1954; Fenner and Ratcliffe, 1965). This suggests that 8 weeks of age may be the ideal time to vaccinate young rabbits born to immune does with Uriarra  $\Delta$ M063Rgptgus, but will require direct testing.
- Detailed histological analysis of rabbit skin following Uriarra  $\Delta$ M063Rgptgus inoculation and after SLS challenge. This may reveal important information regarding the events that result in induction of immunity following vaccination with the replication-deficient virus, and the early events involved in protective immunity upon challenge. Immunohistochemical analysis can be used to identify specific cell types (CD8<sup>+</sup> and CD4<sup>+</sup> T cells, B cells and macrophages) in the skin during vaccination and challenge, and answer questions regarding the specific cell types in the lesion.

### 8.3 Final Summary

The myxoma virus/European rabbit model has provided a means of investigating the efficacy of various novel vaccination strategies against a lethal, naturally transmitted



poxvirus in the species at risk. The results presented here suggest that replication-deficient poxvirus vaccines offer the most promising, highly protective, extremely safe and non-transmissible alternative to currently available poxvirus vaccines. Uriarra  $\Delta$ M063Rgptgus is an excellent candidate vaccine for further development for use in protecting domestic rabbits in Australia from myxomatosis.

# **Appendix**

## **Media, buffers and solutions**

## APPENDIX – MEDIA, BUFFERS AND SOLUTIONS

### Bacterial Growth Media

#### *Luria-Bertani (LB) Broth*

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g

Dissolve in 1 L of MQ H<sub>2</sub>O and autoclave.

#### *LB Agar*

As for LB broth, adding 15 g/L of agar before autoclaving.

#### *Ampicillin*

Add filter-sterilised ampicillin-sodium (100 g/L) to cooled broth or agar to a final concentration of 100 µg/mL.

#### *Kanamycin*

Add filter-sterilised kanamycin (30 g/L) to cooled broth or agar to a final concentration of 30 µg/mL

#### *IPTG / X-Gal Plates*

Add 20 µL each of X-Gal and IPTG solutions (20 mg/mL each) and spread onto the plate.

### Plasmid Preparation

#### *10X STE Buffer*

1 M NaCl
100 mM Tris.HCl pH 8.0
10 mM EDTA pH 8.0

#### *Solution I*

50 mM glucose
25 mM Tris.HCl pH 8.0
10 mM EDTA pH 8.0

Sterilised by autoclaving. Stored at 4°C

#### *Solution II*

0.2 M NaOH
1% SDS

Freshly prepared from sterile stock solutions (10% SDS, 3 M NaOH) before use.

#### *Solution III*

5 M CH <sub>3</sub> COOK	60 mL
Glacial acetic acid	11.5 mL
Water	28.5 mL

Prepared from sterile stock solutions and stored at 4°C

*TE Buffer*

1 M Tris.HCl pH 8.0                      200  $\mu$ L  
0.5 M EDTA pH 8.0                      1 mL  
Make up to 100 mL with MilliQ water and autoclave

**Agarose Gel Electrophoresis**

*20X TAE Buffer*

Tris Base            96.9 g  
EDTA                5.86 g  
Dissolve in 800 mL of MilliQ water. Adjust pH to 8.0 with glacial acetic acid and make up to a final volume of 1 L with water.

*Agarose Gel Loading Buffer*

Glycerol                                      1.2 mL  
10  $\mu$ g/mL Bromophenol blue            2.8 mL  
MilliQ water                                   1 mL

**RNA Gels and Northern Hybridisation**

*DEPC-Treated Water*

2 L water  
2 mL DEPC (Sigma)  
Mix and leave at room temperature for 30 minutes. Autoclave.

*10X RNA Gel Buffer*

200 mM triethanolamine  
50 mM EDTA  
Adjust pH to 7.4.

*Deionised formamide*

500 mL formamide (Sigma)  
50 g Amberlite MBI Resin  
Mix for 20 minutes, then filter to remove resin.

*RNA Sample Buffer*

1 mL deionised formamide  
200  $\mu$ L 10X RNA Gel Buffer  
300  $\mu$ L 37% formaldehyde

*RNA Gel Electrophoresis Buffer*

50 mL 10X RNA Gel Buffer  
80 mL 37% formaldehyde  
370 mL DEPC-treated water

### *30X SSC*

4.5 M Na<sub>3</sub>-citrate

450 mM NaCl

Adjust pH to 7.0. Add 1 mL of DEPC per litre of solution, mix and leave at room temperature for 30 minutes. Autoclave.

### *High-SDS Hybridisation Solution*

De-ionised Formamide 25 mL

30X SSC 8.3 mL

1 M Sodium Phosphate (pH 7.0) 2.5 mL

SDS 3.5 g

10X Blocking Solution (Roche) 10 mL

10% N-laurylsarcosine in H<sub>2</sub>O 500 µL

Heat to 65°C until clear.

### *2X Wash Solution*

2X SSC

0.1% SDS

### *0.5X Wash Solution*

0.5X SSC

0.1% SDS

### *0.2X Wash Solution*

0.2X SSC

0.1X SDS

### *Maleic Acid Buffer*

0.1 M maleic acid

0.15 M NaCl

Adjust pH to 7.5

### *Antibody Wash Buffer*

Maleic Acid Buffer plus 0.3% Tween 20

### *Antibody Blocking/Hybridisation Buffer*

9 parts Maleic Acid Buffer

1 part 10X Blocking Solution (Roche)

## **SDS-PAGE and Western Blotting**

### *4X SDS-PAGE Stacking Gel Buffer*

0.5 M Tris.HCl (pH 6.8)

0.4 % SDS

### *4X SDS-PAGE Resolving Gel Buffer*

1.5 M Tris.HCl (pH 8.8)

0.4 % SDS

*SDS-PAGE Running Buffer*

Tris Base	1.45 g
Glycine	7.2 g
SDS	0.5 g

Dissolve in 500 mL of MQ H<sub>2</sub>O. Do not adjust pH.

*2X SDS-PAGE Gel Loading Buffer*

0.5 M Tris.HCl pH 6.8	1 mL
Glycerol	0.8 mL
10% SDS	1.6 mL
2.5% Bromophenol blue	130 µL
MilliQ H <sub>2</sub> O	3.7 mL

*For reducing conditions add:*

1 M DTT	800 µL
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*Coomassie Blue Staining Solution*

250 mL methanol  
50 mL glacial acetic acid  
250 mL MilliQ Water  
1 g Coomassie Blue powder

*Destaining Solution*

300 mL ethanol  
125 mL glacial acetic acid  
875 mL MilliQ water

*Fixative-1 (Silver Stain)*

40% methanol  
10% glacial acetic acid  
50% MilliQ water

*Fixative-2 (Silver Stain)*

40% methanol  
5% glacial acetic acid  
55% MilliQ water

*Oxidiser Solution (Silver Stain)*

10 mL Oxidiser Solution Concentrate (Bio-Rad)  
90 mL MilliQ water

*Silver Reagent (Silver Stain)*

10 mL Silver Reagent Concentrate (Bio-Rad)  
90 mL MilliQ water

*Developer Solution (Silver Stain)*

32 g Developer Powder (Bio-Rad)  
1 L MilliQ water



*Stop Solution (Silver Stain)*

5% glacial acetic acid  
95% MilliQ water

*Electrotransfer Buffer*

25 mM Tris.HCl  
192 mM Glycine  
0.1% SDS  
15% methanol

*TBS*

NaCl                8 g  
KCl                0.2 g  
Tris Base        3 g  
Dissolve in 800 mL of MQ H<sub>2</sub>O and adjust pH to 8.0 with HCl. Make up to 1 L with MQ H<sub>2</sub>O and autoclave.

*Tween-TBS (TTBS)*

TBS                1 L  
Tween 20        1 mL

*Blocking Buffer (Blotto)*

TBS                                100 mL  
Skim milk powder (Diploma) 10 g

*Antibody Buffer*

TBS                                100 mL  
TTBS                               100 mL  
Skim milk powder               2 g

**Recombinant Protein Purification (TALON IMAC Resin)**

*Extraction/Wash Buffer*

50 mM Sodium Phosphate  
300 mM NaCl

*Imidazole Wash Buffer*

50 mM Sodium Phosphate  
300 mM NaCl  
15 mM Imidazole

*Elution Buffer*

50 mM Sodium Phosphate  
300 mM NaCl  
150 mM Imidazole

## Mammalian Cell Tissue Culture

### Mouse Tonicity Salts

NaCl	11.7 g
Na-Pyruvate	2.2 g
Monothiolglycerol	124 $\mu$ L

Make up to 200 mL with MilliQ water and filter sterilise. Store at -20°C.

### RPMI-1640 (No Additives)

10.4 g RPMI-1640 powder (Sigma)

2.2 g NaHCO<sub>3</sub>

Dissolve in 1000 mL of MilliQ water and filter sterilise. Store at 4°C. If not used within 2 weeks, supplement with 5 mL of 200 mM L-glutamine.

### RPMI(Complete)

RPMI-1640 (No Additives)	500 mL
Foetal Calf Serum (FCS)	50 mL
Penicillin (60 mg/mL) / Streptomycin (100 mg/mL)	500 $\mu$ L
Amphotericin B (250 $\mu$ g/mL)	1 mL
$\beta$ -mercaptoethanol (0.175%)	1 mL

### RPMI (for growth of HT-2 Cells)

RPMI(Complete)	550 mL
Mouse Tonicity Salts	5 mL
Mouse IL-2 (produced in Hi5 cells)	500 $\mu$ L

### Minimal Essential Medium (MEM F<sup>-</sup> A<sup>-</sup>)

9.5 g Minimal Essential Medium powder (Gibco-Invitrogen)

2.2 g NaHCO<sub>3</sub>

Dissolve in 1000 mL of MilliQ water, and filter sterilise. Store at 4°C. If not used within 2 weeks, supplement with 5 mL of 200mM L-glutamine.

### MEM(F<sup>-</sup> A<sup>+</sup>)

To 500mL of MEM(F<sup>-</sup> A<sup>-</sup>), add:

500  $\mu$ L Penicillin (60 mg/mL) / Streptomycin (100 mg/mL)

1 mL amphotericin B (250  $\mu$ g/mL)

### MEM(Complete)

To 500 mL of MEM(F<sup>-</sup> A<sup>+</sup>), add:

50 mL of Foetal Calf Serum

### MEM(Selective)

To 10 mL of MEM(F<sup>-</sup> A<sup>+</sup>), add:

Mycophenolic acid in ethanol (10 mg/mL)	10 $\mu$ L
Xanthine in 0.1 M NaOH (10 mg/mL)	250 $\mu$ L
100x HAT supplement in Milli Q water	100 $\mu$ L

*Trypsin Diluent*

NaCl	8 g
KCl	0.4 g
Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O	0.15 g
KH <sub>2</sub> PO <sub>4</sub>	0.06 g
K <sub>2</sub> EDTA	0.109 g
NaHCO <sub>3</sub>	0.5 g

Dissolve in 1 L of MilliQ water and adjust pH to 7.2. Autoclave.

*ACK Shocking Buffer*

NH <sub>4</sub> Cl	8.29 g
KHCO <sub>3</sub>	1 g
Na <sub>2</sub> EDTA	37.2 mg

Dissolve in 1 L of MilliQ water and adjust pH to 7.3. Autoclave.

*Phosphate Buffered Saline (PBS)*

NaCl	8 g
KH <sub>2</sub> PO <sub>4</sub>	0.24 g
Na <sub>2</sub> HPO <sub>4</sub>	1.44 g
KCl	0.2 g

Make up to 1 L with water and sterilise by autoclaving.

*Methanol:Acetone*

50 mL methanol  
50 mL acetone

## ELISA

*Carbonate Buffer*

Na <sub>2</sub> CO <sub>3</sub>	1.59 g
NaHCO <sub>3</sub>	2.93 g

Dissolve in 1 L MilliQ water and store at 4°C.

*ELISA Blocking Buffer*

PBS with:  
5% skim milk powder  
0.05% Tween 20

*Tween-PBS*

PBS	1 L
Tween 20	5 mL

*ELISA Antibody Binding Buffer*

100 mL Tween-PBS  
1g skim milk powder

*Citrate Phosphate Buffer*

1 M citric acid (pH4.0)            3.07 mL

1 M Na<sub>2</sub>HPO<sub>4</sub> (pH 4.0)            3.85 mL

Make up to 50 mL with MilliQ water.

*HRP Substrate Solution*

10 mL Citrate Phosphate Buffer

1 ABTS substrate tablet (Sigma, Australia)

20 µL 30% H<sub>2</sub>O<sub>2</sub>

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